

AMINO ACID RACEMASE RESPONSIBLE FOR THE BIOSYNTHESIS OF D-
ASPARTATE AND D-SERINE SIGNALING MOLECULES IN APLYSIA CALIFORNICA
CENTRAL NERVOUS SYSTEM

BY

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DISSERTATION

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ABSTRACT

While dogma states that animals use L-amino acids, several D-amino acids have been found in animals including humans since the 1980s. Two D-amino acids have attracted significant research interests because of their cell-cell signaling functions in animal nervous systems. D-serine (D-Ser) can affect the activity and plasticity of N-methyl-D-aspartate (NMDA) receptor, a molecule that forms the basis of learning and memory in the brain. D-aspartate (D-Asp) is found in neuroendocrine and endocrine tissues, potentially playing roles in hormone synthesis and/or release. However, many details on the physiological functions of D-Ser and D-Asp and their signaling pathways remain unknown. Extensive studies are needed to fully understand the roles of these two putative neuronal signaling molecules and their effects on the animal brain and endocrine functions.

D-Ser and D-Asp biosynthetic enzymes are essential to study the physiological functions of the D-amino acids in animal nervous systems. However, the knowledge of such enzymes has been limited, especially the characterization of D-Asp synthetic enzymes. Although the first enzyme that can make D-Ser from L-Ser was isolated from animal brain tissue in 1999, the first nervous system associated D-Asp synthetic enzyme was not described until 2010. The lack of the knowledge of a D-Asp biosynthetic enzyme has significantly hindered the research of D-Asp physiological function in animal nervous system. The molecular mechanism underlying D-Asp action is still not clear. In invertebrates, high levels of D-Asp had been

found in central nervous systems (CNS) of some marine mollusks including *Aplysia californica*, a model animal for cellular and systemic neuroscience study, and D-Asp showed neurotransmitter behaviors in *Aplysia* CNS, indicating D-Asp signaling pathway is conserved among animal kingdoms. Thus, studying D-Asp in *Aplysia* can provide valuable insights into mammalian system function.

As the biosynthetic pathway of D-Asp in invertebrate is not known, one significant goal of this project has been to uncover the biosynthetic enzyme responsible for the biosynthesis of D-Asp in cerebral ganglion in *Aplysia* CNS. Using combining bioinformatics, molecular biology and biochemical methods, a d-amino acid racemase 1 (DAR1) has been discovered, cloned and characterized from the *Aplysia* CNS. The biosynthesis of D-Ser and D-Asp in *Aplysia* CNS cerebral ganglion F/C neuronal clusters via this enzyme has been characterized; these are the regions where high levels of D-Asp were previously detected. DAR1 is the first serine racemase and the first nervous system associated aspartate racemase described from an invertebrate system, and it is also the first enzyme with a dual serine and aspartate activity described in a eukaryotic system. The enzyme thus becomes a valuable model to study D-Ser and D-Asp physiological functions in a well-characterized model system, to investigate D-Ser and D-Asp signaling pathways and their interactions, and to examine the structure-function relationships of pyridoxal-5'-phosphate (PLP) dependent amino acid racemase.

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CHAPTER I

INTRODUCTION

1.1 D-amino Acid

It is known that every amino acid, with an exception of glycine, has two chiral isoforms, L-form and D-form, around alpha carbon chiral center. The enantiomers can convert among each other under extreme temperature or pH spontaneously, by chemical racemization during food processing (1) or by enzymatic racemization (2). For reasons still not clear to scientists, L-amino acids dominate in nature as building blocks of proteins and free molecules with various functions in organisms. D-amino acids, however, are widespread in bacteria where they can be found in bacterial cell walls in the form of peptidoglycan and antibiotics (3), and can regulate cell wall remodeling and cause biofilm dispersal as discovered recently (4). Contrary to the long-standing belief that D-amino acids are not present in higher organisms, free D-amino acids have been found during recent years in yeast, plants and animals including mammalian brains and endocrine tissues as endogenous molecules and play important physiological functions (5). The breakthrough of D-amino acid study in higher organisms came in 1990s when a series of study revealed that D-serine (D-Ser) was an endogenous molecule in rat brain as a co-agonist of N-methyl-D-aspartate receptor (6-8). Since then, D-amino acid in eukaryotic systems has become a new discipline of study both in basic research and application sciences with an increasingly growing pace. A few topics of D-amino acid study are related to the major topics of this work involving amino acid racemase and thus are

reviewed in the next few passages. These topics include D-Ser, D-aspartate (D-Asp), invertebrate D-amino acid study with *Aplysia* and D-Ser and D-Asp biosynthetic racemases.

1.2 D-Serine

D-serine was first found at a significant quantity in rat brain and serum using HPLC method in early 1990s (9), later found in rat forebrain and concentrated in hippocampal dentate gyrus astrocytes, and shown to bind to the glycine binding site of N-methyl-D-aspartate receptor (NMDAR) (7). Subsequent studies proved that D-Ser was an endogenous molecule made from L-Ser via serine racemase (SR) in astrocytes (8) and that it modulates NMDAR activity (6). It is now known that D-Ser is mainly made in neurons (10) and can be released from brain neurons (11), astrocytes (12, 13) and retinal glial cells (14). D-Ser can be uptaken by glial cells and neurons by alanine-serine-cysteine transporter 1 (ASCT1) and ASCT2 (15, 16). D-Ser appears to be shuttled between neurons and glial cells to serve its neuromodulator function in neurons but uptake and stored in glial (10). D-amino acid oxidase (DAAO) plays a major role in biodegradation of D-Ser (17). D-Ser is now accepted as a novel small molecule neurotransmitter/neuromodulator. NMDAR activation requires both glutamate agonist bound to its NR2 subunit and co-agonist D-Ser bound to its NR1 subunit (10). The NMDAR is the basis of learning and memory, affecting brain development and plasticity (18), thus abnormal D-Ser metabolism can affect brain functions. It has been documented that an increased level of D-Ser and a decreased level of D-Ser in brain have been linked to neurodegenerative

diseases amyotrophic lateral sclerosis (19) and schizophrenia (20, 21), respectively. Therefore, the study of D-Ser is not only important to understand NMDAR functions but also important for searching for therapeutic targets for neural disorders.

1.3 D-aspartate

D-aspartate is another well studied D-amino acid in animals. The amino acid has shown some signaling functions in hormone regulations. However, many details about D-Asp are not clear and the molecule remains enigmatic.

D-Asp was first detected in plants and animals using D-aspartate oxidase (DAO) and HPLC in 1960s (22). Since then, D-Asp has been reported in almost every animal phyla (23) and in various tissues including brain (24, 25), retina (26), kidney and liver (27), endocrine tissues such as pineal gland and pituitary (28-30), adrenal gland (31, 32) and testis (33), and exocrine tissues (34, 35). D-Asp showed a peculiar temporal pattern during embryo development. It occurred at high levels in embryonic brain and retina of mammals and birds but quickly dropped to trace levels after birth (36, 37). In rat brain, D-Asp immunoreactivity was observed in distinct nerve cell population and its localization was almost the inverse of D-aspartate oxidase (DAO) (31), suggesting the DAO involves the regulation of D-Asp content.

The physiological roles of D-Asp in hormone synthesis and/or release in various endocrine tissues have been well documented. D-Asp can suppress melatonin release from pineal gland

(38), but it can stimulate prolactin and luteinizing hormone secretion from anterior pituitary (39, 40), and testosterone from testes (41) but inhibit oxytocin from posterior pituitary (42). Studies also found that D-Asp plays some roles in hippocampal neurogenesis (43) and the maturation of adrenal gland (32). However, the molecular mechanism(s) underlying D-Asp physiological function remains unclear. Studies have produced a complex profile for D-Asp. A pharmacology study demonstrated the binding of D-Asp to NMDA-type receptor in rat Harderian gland with a lower affinity than L-glutamate (44). D-Asp seems to have some neuromodulatory effect on NMDAR because an abnormally high level of D-Asp strongly enhanced NMDAR dependent LTP in hippocampal area in a DAO knockout animal model (45). However, a study showed that D-Asp could potentiate L-Glu effects significantly on horizontal cells in goldfish retina (46); another study indicated that D-Asp improved hormone synthesis through cGMP secondary messenger (39). It looked like that D-Asp has more than one function by acting on different receptors, triggering different signaling pathways.

Experiments on intracellular localization of D-Asp with immunohistochemistry (IHC) methods showed conflicting results. D-Asp was found predominantly associated with heterochromatin but undetectable in the other subcellular structures in mammalian hypothalamo-neurohypophyseal system (47) suggesting a gene expression function; but it was located in PC12 (adrenal tumor cell line) and GH3 (pituitary tumor cell line) cytoplasm (48). The discrepancy of the IHC results could be due to staining techniques, antibody quality or different cell types.

Evidences of release and uptake of D-Asp have also been accumulated. It has been shown that D-Asp was released from tissues containing significant levels of D-Asp or loaded with a high concentration of D-Asp to extracellular milieu. For example, D-Asp could be released from adrenal slices by the depolarization of KCl or acetylcholine stimulation (49), from *Aplysia californica* cerebral ganglia upon the stimulation of KCl or ionomycin (50), and from hippocampal nerve endings (51).

The uptake of D-Asp experiments have shown that it could be uptaken by cultured rat pinealocytes (38, 49), astrocytes through an excitatory amino acid transporter (EAAT) (52), PC12 cell through a glutamate transporter-mediated pathway (53) and *Aplysia* cerebral ganglion (50).

The biosynthetic pathway of D-Asp in an animal nervous system was not known until very recently when the first mammalian Asp racemase (DR) was reported in 2010 (43) and the first invertebrate DR was published by us this year (47). The two DRs are distinct enzymes with different catalytic mechanisms. It is clear that there is more than one biosynthesis pathways are involved but how many remains to be answered.

Accumulated evidences so far have depicted D-Asp as a signaling molecule with some features of a classical neurotransmitter like D-Ser. But extensive studies are needed to fully

understand the mechanisms of D-Asp physiological functions in nervous system and/or endocrine systems.

1.4 D-amino Acid study with *Aplysia californica*

Research evidences with invertebrates have indicated that D-Ser and D-Asp signaling pathways are likely conserved among animal kingdoms. D-Ser has been found in *Aplysia californica* CNS (54, 55) and D-Asp found in a variety of tissues in ocean invertebrates CNS (23, 24, 56, 57), endocrine tissues (33, 58) and visual systems (26). Studies with *Aplysia* on D-Asp have pointed a profile of a neurotransmitter. Thus D-amino acid study with *Aplysia* can shine some lights on the function of the amino acid in mammalian systems.

Aplysia californica is a marine mollusk animal model for cellular and systemic neuroscience study. *Aplysia* CNS has many giant and identifiable neurons thus it is a suitable model to study single neuron profiling and neural network. Using analytical instrumentation capillary electrophoresis laser induced fluorescence detection (CE-LIF), D-Asp was first detected in *Aplysia* neuronal clusters and single neurons (59). D-Asp content, expressed as percentage of D-Asp in total Asp, was found distributed unevenly among cerebral ganglion neuronal clusters with the highest content found in F clusters (80 %) and lowest found in neighboring A clusters (40%), suggesting the endogenous origination of D-Asp and a possible physiological function in *Aplysia* CNS (24). The amino acid could stimulate the release of

cardiomodulatory peptide from R3-14 neurons (60), be released from cerebral ganglia in a stimulation dependent manner and uptaken by cerebral ganglion F-clusters, and might be synthesized by a PLP-dependent aspartate racemase activity in cerebral ganglion using radionuclide pulse-and-chase (50). To define D-Asp biological nature in *Aplysia* CNS, it was essential to describe the enzyme that makes D-Asp *in vivo*.

1.5 Significance of D-serine and D-aspartate Biosynthetic Enzymes

The knowledge of biosynthetic pathways of a D-amino acid in a nervous system is essential to define whether a molecule is a neurotransmitter, and a D-amino acid synthetic enzyme is a powerful tool to study the functions of a D-amino acid and its signaling pathway. The discovery of SR and co-localization of SR with D-Ser in rat astrocytes and neurons provided the ultimate proof that D-Ser was made in brain tissues in 1999 and quickly established the physiological function of D-Ser in animal brain (8). However, a decade-delayed discovery of DR in animal nervous system has significantly hindered the research of D-Asp in animal brain functions. Recent cloning of the first mouse DR has helped to discover D-Asp role in hippocampal neurogenesis (43). However, the knowledge of animal DR in nervous system has just started to emerge. Studies with invertebrate system can provide insights into vertebrate systems including human system. Racemase study is the theme of my dissertation research, thus I have dedicated Chapter II to review amino acid racemases extensively.

1.6 Goal and Significance of Research Project

My research project was to find a novel enzyme that is responsible for D-Asp biosynthesis in *Aplysia* CNS cerebral ganglion where exceptionally high levels of D-Asp was previously detected in some isolated neuronal clusters by CE-LIF and where D-Asp behaved like a neurotransmitter. But the biosynthetic enzyme for the D-Asp in *Aplysia* CNS was not known. The discovery of the enzyme provides an evidence for defining the D-Asp as a neurotransmitter/neuromodulator in *Aplysia*. The enzyme would establish *Aplysia* as the first model to study D-amino acid signaling pathway in an invertebrate system. The enzyme can also be used to study the structure and function relationship of eukaryotic racemases and shine some lights on racemase evolution.

1.7 Summary of Research Methods and Dissertation Chapters

A battery of modern biological techniques was used to accomplish *Aplysia* d-amino acid racemase 1 (DAR1) project. Bioinformatics tools were used for searching racemase gene from *Aplysia* genome database and analyzing various protein properties based on its primary sequence. A variety of molecular biology techniques have been used to clone the complete cDNA sequence of *Aplysia* genes, and to construct protein expression vectors. Protein chemistry techniques were used to express and purify proteins from microbial expression systems, and to measure protein quantity and quality. Biochemical enzyme assay, both qualitative and quantitative, were used to describe enzyme characteristics and measure kinetics. Immunology techniques were used to make polyclonal and monoclonal antibodies, and to perform Western blot and IHC to examine protein expression and localization in

Aplysia tissues. Finally, analytical chemistry method CE-LIF was used for analyzing racemase reaction results, which was essential for the success of the project.

The contents of subsequent Chapters are summarized below. Chapter II is a detailed review of amino acid racemase that is the focus of my dissertation topics. Chapter III and Chapter IV cover my major Ph.D. research project on DAR1. In Chapter III, the discovery of DAR1, from gene to enzyme, was described in detail. The discovery has established *Aplysia* as the first invertebrate model to study D-Asp and D-Ser. The contents of this chapter have been published. In Chapter IV, the L-Ser β -eliminase activity of DAR1 was described by side-by-side comparisons with *Aplysia* serine dehydratase AcSDH, a novel and distinct enzyme with the same β -eliminase activity. A new hypothesis was proposed on the basis of the study to explain the physiological function of DAR1 β -eliminase activity. The hypothesis, if confirmed by others, would provide a general answer to the β -elimination activity of eukaryotic serine racemase which has been under debate. The contents of Chapter IV are in the preparation for a journal article submission. Chapter V includes three methods that were used for DAR1 projects but not described in the previous chapters; it also includes the descriptions of two experiments, one of which is generating monoclonal antibody against D-amino acid containing peptide (DAACP), and the other is suprachiasmatic nucleus (SCN) microdialysis fluid analysis for L-glutamate neurotransmitter change using CE-LIF method. The two experiments are not directly associated to the DAR1 project but they are parts of D-

amino acid study and neurotransmitter study which are under the same theme of my dissertation research.

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CHAPTER II

AMINO ACID RACEMASES

2.1 Introduction

Free D-amino acids are essential for bacterial growth as cell wall peptidoglycan components, and they are also present in archaea with putative function related to the uptake of D-amino acids formed under high temperature stress (1). Oddly, the four D-amino acids (D-Ala, D-Ser, D-Asp and D-Glu) found in bacterial cell wall peptidoglycan materials have been discovered as endogenous molecules in various eukaryotic organisms including mammals during recent years and have shown a range of important physiological functions (2-4). In animals, D-Ser is a neurotransmitter/neuromodulator acting on NMDA receptor in CNS (5), D-Asp plays roles in hormone synthesis/release in CNS and peripheral endocrine tissues (6) and D-Ala is implicated affecting insulin secretion from pancreas (7). D-Glu occurs in animal CNS in significant amount but its function has not been revealed (8, 9). It is obvious that D-amino acid studies in animals not only is important in basic science but likely has clinical significance.

The knowledge of D-amino acid biosynthetic enzymes is essential to understand the physiological functions of D-amino acids and D-amino acid signaling pathways in animals. It is known that in bacteria D-amino acids for peptidoglycan syntheses are mainly made by amino acid racemases (1) which can catalyze interconversion between an L-amino acid and its

D-amino acid counterpart. Amino acid racemases are found not only in bacteria but also in archaea, fungi, yeast, plant and animals (2). There are two categories of amino acid racemase. One class requires pyridoxal-5'-phosphate (PLP, or a vitamin B₆ active form) cofactor for activity, and are termed PLP-dependent racemases; another class is cofactor free, or termed PLP-independent racemase. PLP-independent racemases have mostly been found in bacterial and archaea, but PLP-dependent racemase occurs in prokaryotes, archaea and eukaryotes. Both classes of amino acid racemases are found in many bacteria phyla and have been well studied. The knowledge of bacterial D-amino acid biosynthetic pathways has helped to find eukaryotic racemase. So far, about total a dozen of eukaryotic amino acid racemases have been described from yeasts (10, 11), plants (12) and animals (13-15). However, the knowledge of racemase associated with animal brain and/or endocrine tissue is limited and growing slowly. Three serine racemases in mammalian brains were described by 2000, but the first aspartate racemase from an animal nervous system was described in 2010; and so far no alanine and glutamate racemases have been found in animal nervous/endocrine tissues although both D-Ala and D-Glu have been found in CNS in significant amounts. Therefore it is necessary and important to find new racemases from animal nervous systems and use them to study D-amino acids in animals. A recent approach to find a new enzyme is to search annotated target genes or search genomes for a target gene from scratch using known protein sequences or structures. Once a target gene is found, the gene can be cloned and expressed and its protein can be characterized. Therefore the knowledge of known enzymes is very important for discovering and characterizing new enzymes of similar functions. The goal of

this review is to gather information from the amino acid racemases studied so far by describing some representative racemases from bacterial, archaea, yeast, plants or animals. The information can facilitate D-amino acid racemase studies and aid in understanding this elusive and intriguing modification in animals.

2.2 Amino Acid Racemase Catalytic Mechanisms and Structures

2.2.1 PLP-independent racemase

Enzyme structure and catalytic functions are essential to understand an enzyme. As mentioned above, there are two classes of amino acid racemase: PLP-independent racemases and PLP-dependent racemases. Their structures and catalysis mechanisms are distinct. However, the basis of amino acid racemization is the same. It involves the initial removal of α -proton from an amino acid substrate and then a reprotonation of the same substrate but on the opposite face of the resulting planar anionic intermediate. The tasks of α -proton abstraction and addition are performed by enzyme residues for PLP-independent enzymes or performed by enzyme residues but aided by PLP for PLP-dependent enzymes as shown in Figure 2.1. This section describes catalytic mechanism of PLP-independent enzymes using bacterial glutamate racemase as an example.

Cofactor free amino acid racemase either uses one cysteine base to perform both α -proton abstraction and delivery task or use two cysteine bases with one abstraction base and one delivery base for α -proton transfer. The one-base mechanism is used by bacterial proline

racemase (16, 17), and two-base mechanism is used by bacterial glutamate racemase, bacterial aspartate racemases and archaea aspartate racemase (1), and proline racemase from human parasite *Trypanosoma cruzi* (18).

Much knowledge of PLP-independent two-base catalysis mechanism came from the mutation studies of glutamate racemase (EC 5.1.1.3) from bacteria *Pediococcus pentosaceus* (19), and structural studies of glutamate racemase (MurI) from *Aquifex pyrophilus* (20) and *Lactobacillus fermenti* (21). Crystal structure analysis of MurI revealed that the enzyme forms a dimer and each monomer consists of two α/β fold domains, D-glutamine analog binds to the deep pocket formed by conserved residues from two monomers. Two-base catalytic mechanism by *Lactobacillus fermenti* glutamate racemase is shown in Figure 2.2. The racemization catalytic task is achieved by two active-site cysteine residues Cys73 and Cys184 which act as catalytic bases: thiolate of one cysteine serves to deprotonate the substrate, and the thiol of the second cysteine protonates the carbanionic intermediate in the opposite face. The task is difficult because the pK_a of the α -proton of the amino acid is about 21 (22) and that of a thiol is around 10; therefore, in order to act as catalytic bases to withdraw α -proton from the substrate, the thiols need to be assisted by other residues. Crystal studies showed that Asp10 and His186 assisted the functions of Cys73 and Cys184 through hydrogen bonds, respectively (1, 21).

2.2.2 PLP-dependent Racemase

PLP-dependent enzymes catalyze a large variety of chemical reactions mainly involved in amino acid metabolisms. These enzymes have been divided into fold types on the basis of evolutionary relationships and protein structural organization (23). Different fold-types share limited structural similarities even though they appear to catalyze similar reactions; there is no correlation between fold-types and reaction specificities, for example, amino acid racemases have been found in fold-type I, II and III thus considered to be evolved from three divergent pathways (2). Bacterial alanine and serine racemases are PLP-dependent enzymes and all eukaryotic racemases found so far are also PLP-dependent enzymes, thus PLP-dependent catalysis mechanism is more advanced than PLP-independent mechanism.

Bacterial alanine racemase (EC 5.1.1.1) is wide present in bacterial kingdoms and it is an essential enzyme for bacteria growth for it makes D-Ala, an indispensable component of cell wall peptidoglycan. The enzyme is thus a drug target for antibiotics (23) and is the best studied fold-type III PLP-dependent amino acid racemase. The enzyme was first cloned and characterized by Tanizawa et al. from *Bacillus stearothermophilus* (24). The structural studies with the *B. stearothomophilus* alanine racemase revealed a homodimeric enzyme with each monomer consisting of an α/β barrel domain at the N-terminus (residue 1-240) and a C-terminal domain (241-388) composed mainly of β -strand as shown in Figure 2.4. In the structure of the dimer, the mouth of the alpha/beta barrel of one monomer faces the second domain of the other monomer. The PLP cofactor lies in and above the mouth of the alpha/beta

barrel (25). The enzyme uses two-base mechanism as shown in Figure 2.3. The task of α -proton abstraction is carried out by Try265' and Lys39 to remove α -proton from L- and D-alanine, respectively. The hydrogen transfer between Try265' and Lys39 was mediated by carboxylated oxygen atom of L-alanyl-PLP aldimin or D-alanyl-PLP aldimin. In this mechanism, the phenolic hydroxyl group of Try265' removes α -proton from L-alanyl-PLP aldimin and donates it to the carboxylate group of the aldimin, the resulting carboxylate group then donates the proton to the ϵ -amino group of Lys39 which in turn donates it to C α to form the D-alanyl-PLP aldimine. PLP directly interact with alanine to form Schiff base and become quinonoid intermediate after α -proton was removed and it not only forms the intermediate but stabilizing it (1). The similar mechanism is used by bacterial serine racemase VanT from *Enterococcus gllinarium* where PLP-binding Lys371 and Try597 remove α -hydrogen from L-Ser and D-Ser, respectively (26).

Eukaryotic serine racemase (EC 5.1.1.18) was first isolated from rat by Wolosker et al. (27). It is the best studied eukaryotic racemase and human serine racemase is now a potential drug target for schizophrenia (28). The enzyme has a different structure from alanine racemase and it belongs to fold-type II PLP-dependent enzyme as shown in Figure 2.4. The crystal structures of fission yeast, human and rat serine racemases have been described (11, 29). The structures have revealed a typical fold-type II PLP-dependent enzyme family with both a large domain and a flexible small domain associated into a symmetric dimer, and indicated a ligand-induced rearrangement of the small domain that organizes the active site for specific

turnover of the substrate (11, 29). PLP-binding Lys57 (yeast), and PLP-stabilizing residues, Asn84 (yeast), Glu281 (yeast) and Asn311 (yeast) are conserved in all eukaryotic SR discovered so far (11). Eukaryotic serine racemase is structurally similar to *E. coli* biosynthetic L-threonine dehydratase (EC 4.2.1.16). The N-terminal domain (1-320) of the *E. coli* enzyme contains a PLP attachment motif and the domain shares 31% sequence identity with mouse serine racemase of 334 amino acids.

Another type of PLP-dependent amino acid racemase is represented by mouse aspartate racemase (30). The enzyme is distinct from alanine racemase (prokaryotes or eukaryotes) and serine racemase (eukaryotes) both in catalytic mechanisms and the structure. It is conserved with aspartate aminotransaminase (2.6.1.1). There is no structural information on the enzyme but it is likely belongs to fold-type I PLP-dependent enzyme (2).

2.3 Bacterial and Archaea Racemases

D-Ala is an essential cell wall component across bacterial kingdom and alanine racemase is widespread in bacteria. Two distinct alanine racemase genes have been cloned from *Salmonella typhimurium* termed *dadB* and *alr*, but only *alr* enzyme plays role in peptidoglycan assembly, both enzymes are PLP-dependent and share large homology (31). Some bacteria have homolog of both genes but others have homolog of one gene (1).

D-Glu is strictly conserved across bacterial kingdom occurring in peptidoglycan second residue incorporated. The amino acid is vital for bacterial growth and is mostly synthesized by cofactor free and two-thiol-based glutamate racemase; the conserved and essential feature of glutamate racemase across bacterial kingdom makes it a drug target for anti bacterial agents (32).

D-Asp also occurs in the peptidoglycan of some bacterial strains. Aspartate racemase (EC 5.1.1.13) has been demonstrated in various *Lactobacillus* and *Streptococcus* strains (33). Like bacterial glutamate racemase, bacterial aspartate racemase is also PLP-independent enzyme using cysteines as catalytic bases (1). But the enzyme is less spread among bacteria than glutamate racemase.

Bacterial serine racemase only occurs in some strains. It is PLP-dependent enzyme and shares conserved PLP-binding Lys and Try residues with alanine racemase at its C-terminus. The enzyme plays an important role in vancomycin resistance by modifying peptidoglycan precursor from D-Ala-D-Ala to D-Ala-D-Ser (34, Arias, 1999 #17). The enzyme shares little similarity to eukaryotic serine racemase.

Archaea contains significant free D-enantiomers of serine, alanine, proline, glutamate and aspartate (35) but the physiological functions of the D-amino acids are not clear. PLP-independent archaea aspartate racemase have been isolated from archaea *Desulfurococcus* and

Thermococcus (33, 36). *Desulfurococcus* enzyme shares high homology with bacterial glutamate racemase around putative cysteine catalytic sites. The enzyme might serve a function of uptake of Asp formed under high temperature or producing D-Asp for cell component (33, 36). Crystal structure analysis with mutants and molecular dynamics simulations revealed that archaea DR also use two cysteines as catalytic bases (37, 38).

It is worth describing several details related to the archaea serine racemase from *Pyrobaculum islandicum* (39). The enzyme has an increased activity between 20 °C and 100 °C. Archaea serine racemase is a PLP-dependent enzyme and shares 30% sequence identity to mouse serine racemase (will be described below), and 34% and 29% identity to *E. coli* threonine dehydratase catabolic and biosynthetic enzyme, respectively. It also contains a conserved motif of SFKIRG among serine/threonine dehydratase from human, rat, yeast and *E. coli* and share 66% homology to other archaea threonine dehydratase in primary sequence. The archaea enzyme is similar to both eukaryotic serine racemase and serine/threonine dehydratase in enzyme characters. The enzyme has broad racemase substrate specificities towards L-Ser, L-Thr, D-Ser and D-Thr while mammalian serine racemases have no threonine racemase activity. Both archaea and mammalian serine racemases have dehydratase activity towards L-Ser and L-Thr and their dehydratase activities are much stronger than their racemase activities. However, mammalian serine racemase is activated by ATP-Mg²⁺ but archaea serine racemase is inhibited by ATP and activated by AMT and no response to Mg²⁺ and Ca²⁺. The response to ATP and Mg²⁺ is similar to serine/threonine dehydratase from

Aplysia (see Chapter IV for AcSDH data). The physiological function of the archaea enzyme is not clear but it is not involved in ATP or isoleucine production (39). From the phylogenetic tree as shown in Figure 2.6, it appears that archaea serine racemase is closer to *E. coli* threonine dehydratase than eukaryotic serine racemase clusters and it could be a distant ancestor of eukaryotic serine/aspartate racemases. It is interesting that *Aplysia* racemase DAR1 (will be described below) is heat stable even above 50 °C, a possible trace feature of an ancestor enzyme living under high temperature.

2.4 Eukaryotic Racemase

2.4.1. Serine Racemase

Serine racemase is responsible for D-Ser biosynthesis in mammalian brains (40). Because D-Ser is a co-agonist to NMDA receptor, the aberrant metabolism of D-Ser can affect the function of the key receptor for learning and memory as accumulated evidences have indicted (41). The important physiological function of D-Ser has made serine racemase the most studied eukaryotic amino acid racemase in animals. Human serine racemase is a target for drug development for neurodegenerative disorders such as schizophrenia and ALS (42).

Eukaryotic serine racemases have been cloned and characterized from 7 organisms including fission yeast *S. pombe* (43), plant *A. thaliana* and *H. vulgare* (12, 44), mollusk *A. californica* (15), and human, rat and mouse (13, 27, 45). Putative SR genes are widespread in animal genomes. The enzymes are highly conserved and contain 323-340 amino acid residues and

share more than 37 % protein sequence identity to each other with a conserved PLP-binding motif at N-terminus around PLP-interacting Lys57 (yeast) and PLP-stabilizing residues, Asn84 (yeast), Glu281 (yeast) and Asn311 (yeast) as shown in Figure 2.5 multiple sequence alignment in Fig. 2.5. Human SR gene contains seven exons and localizes to chromosome 17q13.3 (13); *Aplysia dar1* gene discovered by us is composed of 26,276 nucleotides and contains 9 exons. Rat and mouse SR have a serine deletion compared to human homolog; and rat SR seems to have a nonsense mutation that occurred at 334th codon thus terminated earlier than mouse and human SR, leading to a 6 amino acid truncation at C-terminus (46).

The crystal structures of human, rat and fission yeast serine racemases have been described. The structures revealed a typical fold-type II of PLP-dependent enzyme family (11, 29). The enzymes catalyze interconversion between L-Ser and D-Ser with smaller K_m to L-Ser substrate but larger V_{max} towards D-Ser. *Aplysia* DAR1 also has significant aspartate racemase activity (15). The racemization kinetics parameters, especially V_{max} vary significantly from one enzyme to the other, with about as much as 90 folds difference. For example, for L-Ser racemase reactions, the V_{max} of *A. thaliana* SR and *A. californica* DAR1 are 2.0 nmol/mg/min and 11 μ mol/mg/h respectively (15, 44). The difference might be due to the different assay conditions and/or product detection methods used to measure the kinetics. With no exception, however, all serine enzymes also have β -eliminase activity capable of degrading L-Ser, or L-Thr and D-Ser in some cases, to α -keto acids and ammonia. L-Ser β -eliminase activity is usually 2-4 folds stronger than serine racemase activity (44, 47). Both racemase and

dehydratase activities favor alkaline conditions with racemase activity more so than dehydratase activity (15, 48). The enzymes, except for plant SR, are activated by ATP-Mg²⁺ allosteric effectors. However, ATP was not hydrolyzed during human SR activation (49). The physiological function of mammalian SR racemase is to make NMDAR co-agonist D-Ser, but β -elimination activity of serine racemases is under debate which is discussed extensively in Chapter IV.

The intercellular and intracellular localizations of animal serine racemase have been observed. Rat serine racemase is located in both glial cells and neurons confirmed by immunohistochemistry staining technique and in the cytoplasm of cells (41). But whether the enzyme is in some subcellular vesicles is not known. Given the fact that animal serine racemase needs an alkaline condition to achieve a maximal catalytic rate, the enzyme might act in some basic subcellular compartments or by other mechanisms unknown. One possible mechanism is to raise pH surrounding enzyme catalytic pocket by ammonia produced by racemase β -elimination activity. Serine racemase also exists in peripheral tissues. Northern blot analysis found that human SR is expressed in brain, heart, skeletal muscle, kidney and liver tissues with at least three different sizes of mRNA transcripts present in heart and kidney; and the SR was also localized by Western blot in hippocampus, thalamus, and amygdala glial cells (50). In *Aplysia*, Western blot localized DAR1 in brain, liver and atrial gland as a single band of predicated size but a larger protein band was present in ovotestis besides a very faint band of the predicated size (15). *Aplysia* atrial gland is known to secrete a family of peptides

that can stimulate egg laying (51), suggesting that DAR1 might play a role in the hormone synthesis/release in the atrial gland and possible through D-Asp made by DAR1 aspartate racemase activity.

Mammalian serine racemase regulation has been studied. Rat serine racemase is activated by glutamate transmission via glutamate interacting protein which binds to α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subtypes of glutamate receptor. The binding of the serine racemase to the glutamate receptor binding protein augmented enzyme activity which leads to the enhancement of neonatal cerebellum granule cell migration (52). Serine racemase activity is inhibited upon S-nitrosylation by neuronal nitric-oxide synthase, which might be one of the mechanisms modulating NMDA neurotransmission long term potentiation via nitric oxide (NO) in a retrograde manner (53).

2.4.2 Aspartate Racemase

D-Asp has shown physiological functions in hormone synthesis/release and neurogenesis. But the mechanisms underlying D-Asp function is not defined. Studies have depicted a complex picture of a signaling molecule. Aspartate racemase makes D-Asp in vivo (15, 30) but the knowledge of aspartate racemase in animal nervous systems just started to emerge. The first enzyme in an animal nervous system was not described until 2010. The scarce information on aspartate racemase from a nervous system and/or an endocrine system has hindered the study

of D-Asp function in those tissues. Extensive studies are needed to study described enzymes and discover new aspartate racemases from animal nervous and endocrine systems.

So far, only three total aspartate racemases have been described from eukaryotes including two mollusk enzymes and one mouse enzyme. All three enzymes are PLP-dependent but represent two distinct enzyme families based on catalytic mechanisms. Mollusk *S. broughtonii* aspartate racemase (EC 5.1.1.13) from foot muscle and *A. californica* DAR1 (the enzyme also has serine racemase activity) from CNS are very close to each other sharing 55% sequence identity, and to serine racemase family sharing larger than 40% of sequence identity. Mouse serine racemase, however, belongs to aspartate aminotransferase (AST) family (EC 2.6.1.1) with less than 15% sequence homology with Sb DR and DAR1. It is not known if Sb DR is present in bivalve brain but DAR1 and mouse DR occur in animal nervous systems. It is clear now that there are at least two different aspartate racemases that can make D-Asp from L-Asp in animal brains. Based on the structure and catalytic mechanisms, two aspartate racemases have evolved from two divergent pathways (see Figure 2.6 phylogenetic tree). How many enzymes involved in making D-Asp is unknown. But enzymes that make D-Asp in various animal tissues must be present. By careful analyzing known enzymes that can make D-Asp in bacteria, archaea and eukaryotes can provide clues to find new aspartate racemase from animal genomes.

There is no structural information on PLP-dependent aspartate racemase. However, based on sequence homology and highly conserved catalytic residues between serine racemase and mollusk aspartate racemases, it can be assumed that the marine animal aspartate racemases belong to fold-type II PLP-dependent enzymes. The mouse DR is likely a fold-type I PLP-dependent enzyme because aspartate aminotransaminase is fold-type I enzyme (23). Both Sb DR and DAR1 have Asp racemase activity but DAR1 also has serine racemase activity that Sb DR is inactive on serine. Both enzymes display a smaller K_m for L-Asp but larger V_{max} for D-Asp substrate reactions, meaning L-Asp is likely a substrate *in vivo*; and like all serine racemase, both enzymes have L-Ser dehydratase activities (14, 15). However, Sb DR and DAR1 might have different physiological functions *in vivo* because Sb DR is inhibited by ATP in a non-competitive manner and promoted by AMP (54) while DAR1 is opposite (see Chapter IV data); Sb DR does not need Mg^{2+} to activate (55) but DAR1 is strongly activated by ATP- Mg^{2+} (15), indicating that the two enzymes play different physiological roles. It is known that *Aplysia* genome does not contain any other gene that is closer to Sb DR than *dar1*, suggesting that different aspartate racemase can have different function in different tissues in marine mollusks. Mouse DR plays some roles in hippocampal neurogenesis (30).

2.4.3 Alanine Racemase

Free D-alanine has been found in aquatic crustaceans and bivalve mollusks (56), insect (57), plant seeds (58), mammalian brain anterior pituitary (7, 59) and pancreas (60). Research has

indicated that D-Ala is responsible for the intracellular isosmotic regulation in the tissues of crustaceans and bivalves under high salinity stress (56). Alanine has shown to colocalize with insulin secreting β -cells in pancreas (60) and correlate with circadian cycle with high levels during sleeping period and low levels during active period in rats with both diurnal and nocturnal habits (7). Alanine racemase have been cloned from prawn *Marsupenaeus japonicus* muscle and hepatopancreas (61), and from fission yeast (10). Protein sequence homology analysis concluded that eukaryotic and prokaryotic alanine racemases are conserved PLP-dependent enzymes and with similar catalytic residues (61). D-Ala physiological function in animals has not been defined. So far no alanine racemase has been isolated from vertebrates or from a nervous system where significant amount of D-Ala has been found. More studies are needed to find such enzymes to facilitate study of D-Ala in animals.

2.4.4 Proline racemase

Free D-proline (D-Pro) has been found in significant amounts in mouse anterior pituitary, posterior pituitary and pineal glands. In the peripheral tissues, the amounts of D-Pro were high in the pancreas and kidney, and at least some D-Pro is endogenously made (62), thus D-Pro might have some biological functions in mammals. However, no proline racemase has been discovered from animal systems except from human parasite *Trypanosoma cruzi* (63). The *T. cruzi* proline racemase, however, is a PLP-independent amino acid racemase and is the only PLP-independent enzyme being found in eukaryotes so far. In contrast to bacterial

proline racemase, which uses one cysteine base mechanism (17), the *T. cruzi* proline enzyme uses two-cysteine base catalytic mechanism (18). The parasite enzyme has received a significant amount of attention because it can trigger host B cell polyclonal activation, which prevents specific humoral immune responses, and is crucial for parasite evasion and persistence in the human host. In addition, the enzyme is responsible for free D-proline biosynthesis. The function of free D-Pro is not known in the parasite but D-Pro is present in some peptides in non-infective epimastigote and infective metacyclic parasite forms. The D-Pro-containing peptide might be beneficial for resistance to degradation by host protease (64).

The study of D-Pro in animals is just beginning. The discovery of proline racemase will advance the study of D-Pro in the neuroendocrine tissues where significant amount and endogenous D-Pro has been found. As of now, not only little is known about the racemase, the function of this D-amino acid remains enigmatic.

2.5 Figures

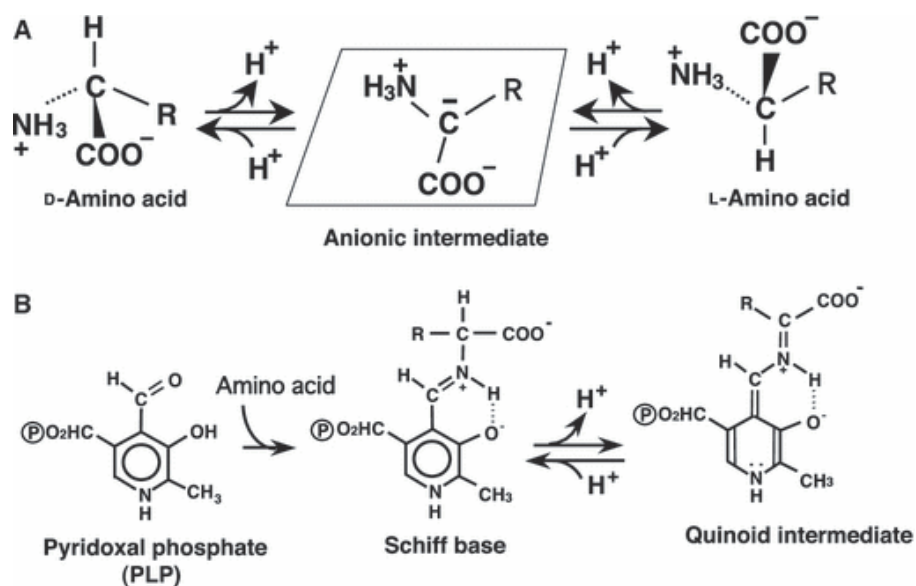


Figure 2.1 Racemization of amino acid (A) and the role of PLP (B). Reproduced with permission from Yoshimura and Goto (2).

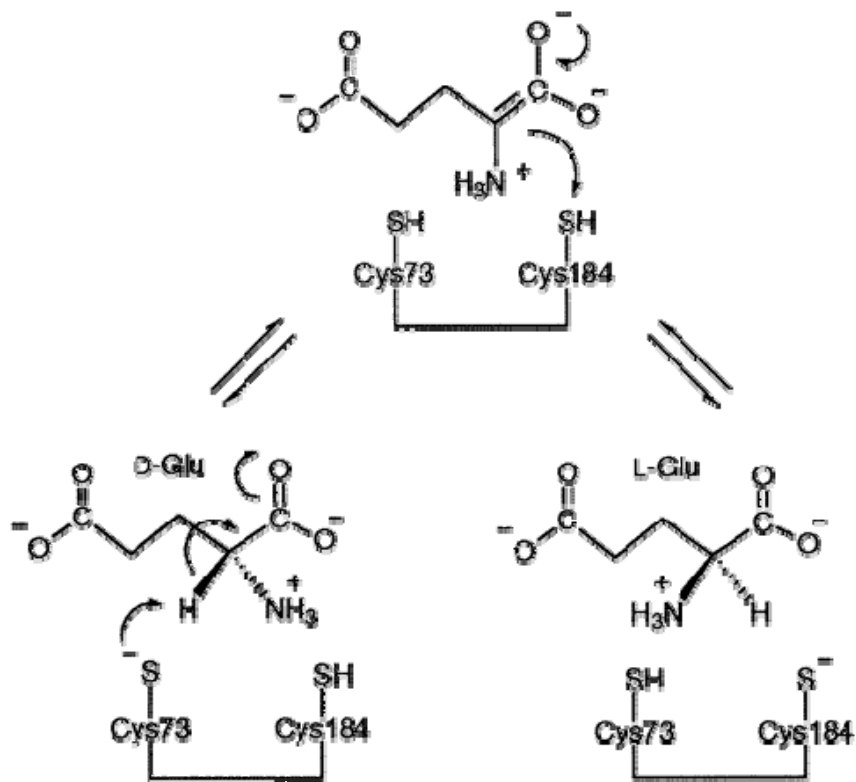


Figure 2.2 Catalytic mechanism of PLP-independent glutamate racemase from *L. fermenti*. Reprinted with permission from Tanner et al.(65).

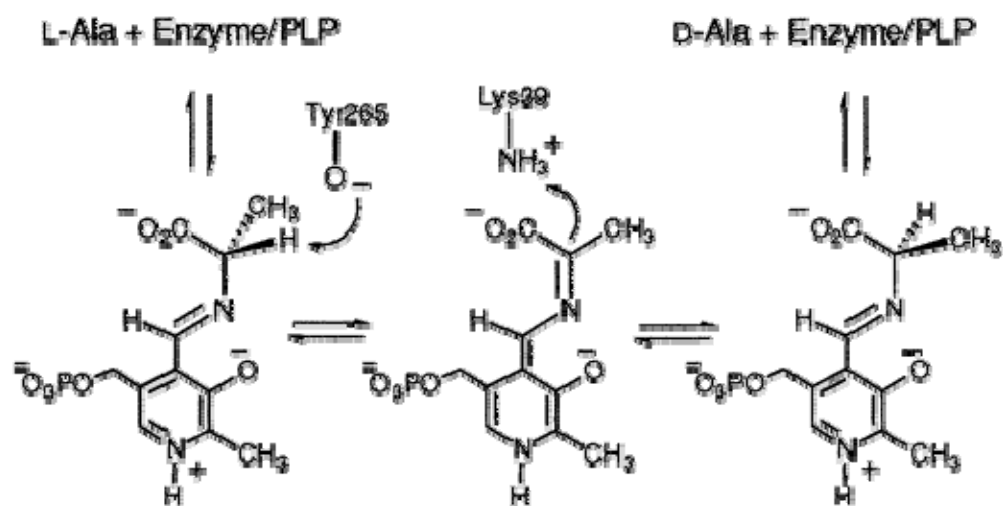


Figure 2.3 Catalytic mechanism PLP-dependent alanine racemase from *B. stearothermophilus*. Reprinted with permission from Tanner et al. (65) .

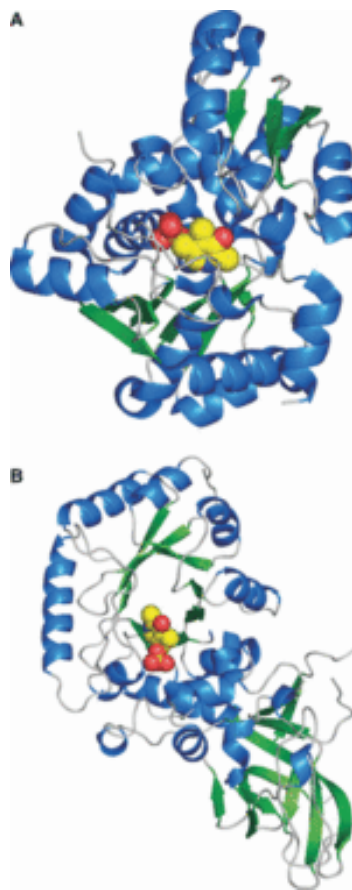


Figure 2.4 Structures of two PLP-dependent amino acid racemase. *S. pombe* serine racemase (fold-type II) (A) and *G. stearothermophilus* alanine racemase (fold-type III) (B). Figure reproduced with permission from Yoshimura and Goto (2).

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Mouse_SR      -----MCAQYCISFADVEKAHINIQDSIHLTPVLTSSILNQIAGRNLFFKCELF
Rat_SR        -----MCAQYCISFADVEKAHLNIQDSVHLTPVLTSSILNQIAGRNLFFKCELF
Human_SR      -----MCAQYCISFADVEKAHINIIRDSIHLTPVLTSSILNQLTGRNLFFKCELF
At_SR         -----MEANREKYAADILSIKEAHDRIKPYIHRTPVLTSESLNSISGRSLFFKCECL
Hv_SR         MGSRDDDDGHSTEGGYAADINSIREARARIAPYVHKTPILSSTSIDAIAIGQLFFKCECF
Aplysia_DAR1  -----MAASCGVTFVDVLKALERISPFIHKTPIFTSEQANRKCGRQLFFKCECF
yeast_SR      -----MSDNLVLPTYDDVASASERIKKFANKTPVLTSSSTVNKEFVAEVFFKCECF
               .:.*.*.*.:*****:

Mouse_SR      QKTGSFKIRGALNAIRGLIPDTPEEKPKAVVTHSSGNHGQALTYAAKLEGIPAYIVVPQT
Rat_SR        QKTGSFKIRGALNAIRGLIPDTLEGKPKAVVTHSSGNHGQALTYAAKLEGIPAYIVVPQT
Human_SR      QKTGSFKIRGALNAVRSLVPDALERKPKAVVTHSSGNHGQALTYAAKLEGIPAYIVVPQT
At_SR         QKGGAFKFRGACNAVLSLD---AEQAAKGVTTHSSGNHAAALSLAAKIQGIPIAYIVVPKG
Hv_SR         QKAGAFKIRGASNSIFALD---DSQAAKGVTTHSSGNHAAAVALAACLGRIPAYIVIPKN
Aplysia_DAR1  QKSGSFKARGALNAVLCQ--QVKPNVNGVTTHSSGNHGQALAWAAQRANLPCCVVVPQM
yeast_SR      QKMGAFFKRGALNALSQLN---EAQRKAGVLTFFSSGNHQAIALSAKILGIPAKIIMPLD
               ** **.* ** **.:.*.******.*::*:.*.:**.:**

Mouse_SR      APNCKKLAIQAYGASIVYCDPSDESREKVTQRIMQETEGILVHPNQEPAVIAGQGTTIALE
Rat_SR        APNCKKLAIQAYGASIVYSEPSDESRENVQRIIQETEGILVHPNQEPAVIAGQGTTIALE
Human_SR      APDCKKLAIQAYGASIVYCEPSDESRENVAKRVTEETEGIMVHPNQEPAVIAGQGTTIALE
At_SR         APKCKVDNVIRYGGKVIWSEATMSSREEIASKVLQETGSVLIHPYNDGRIISGQGTIALE
Hv_SR         APACKVENVRRYGGQVIWSDVTMESRESIAKKVQETGAILIHPFNDKYTISGQGTVCLE
Aplysia_DAR1  APDVKKNAIRGYGAELLECGPKPSDRNEACDKVQDDRNFEIIPYDHDVVIAGQGTTIAVE
yeast_SR      APEAKVAATKGYGGQVIMYDRYKDDREKMAKEISEREGLTIIPYDHPHVLGQGTAAKE
               ** * **.*.:**.*.:**.:**.:**.:**.:**.:**.:**.:**.:**

Mouse_SR      VLNQVPLVDALVVPVGGGGMVAGIAITIKALKPSVKVYAAEPSN--ADDCYQSKLKGELT
Rat_SR        VLNQVPLVDALVVPVGGGGMVAGIAITIKTLKPSVKVYAAEPSN--ADDCYQSKLKGELT
Human_SR      VLNQVPLVDALVVPVGGGGLAGIAITVKALKPSVKVYAAEPSN--ADDCYQSKLKGKLM
At_SR         LLEQIQEIDAIVVPISGGGLISGVALAAKSIKPSIRIIAAEPKG--ADDAQSKVAGKII
Hv_SR         LLEQVPEIDTIIIVPISGGGLISGVTLAAKAINPSIRILAAEPKG--ADDSAQSKAAGRII
Aplysia_DAR1  LLEQVPFLDAILVPISGGMSSGICIAAKTIKPDIKIFIVAPKGRLEECLRTGKRWPWE
yeast_SR      LFEEVGPLDALFVCLGGGGLSGSALAARHFAFNCEVYGVPEA--GNDGQSFRRKSIV
               ::::.*::.*::.*::.*::.*::.*::.*::.*::.*::.*::.*::.*::

Mouse_SR      PNLHPPETIADGVK--SSIGLNTWPIIRDLDV-DVFTVTEDEIKYATQLVWGRMKLLIEPT
Rat_SR        PNLHPPETIADGVK--SSIGLNTWPIIRDLDV-DVFTVTEDEIKYATQLVWERMKLLIEPT
Human_SR      PNLYPPETIADGVK--SSIGLNTWPIIRDLDV-DIFTVTEDEIKCATQLVWERMKLLIEPT
At_SR         T-LPVTNTIADGLR-ASLGDLTWPVVRDLVD-DVVTLEECEIIEAMKMCYEILKVSVEPS
Hv_SR         K-LPATSTIADGLR-AFLGDLTWPVVRDLVD-DVIVVDDNAIVDAMKMCYETLKVAVEPS
Aplysia_DAR1  P-PQYLDTIADGIRLQQTGYITTPILMELAEKDVFEMSDEEIEGMKFSFERMKLVIETA
yeast_SR      H-IDTPKTIADGAQTQHLGNYTFSIIKEKVD-DILTVSDEELIDCLKFYAARMKIVVEPT
               .*****: * *.:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:

Mouse_SR      AGVALAAVLSQHFTQTVS--PEVKNCIVLSGGNVDLTS-LNWVGQAERPAPYQTVSV
Rat_SR        AGVGLAAVLSQHFTQTVS--PEVKNCIVLSGGNVDLTS-LSWVKQAERPAP-----
Human_SR      AGVGVAAVLSQHFTQTVS--PEVKNCIVLSGGNVDLTSITWVKQAERPASYQSVSV
At_SR         GAIGLAAVLSNSFRNPNPSCRCKNIGIVLSGGNVDLGLSLWDSFKSSK-----
Hv_SR         GAIGLAAALSDEFKQSSAWHESKIGIIVSGGNVDLRVLWDSLYK-----
Aplysia_DAR1  AGASVAAAFSDRLRKMD--PDLKNVGVILCGGNLDIENLFP-----
yeast_SR      GCLSFAAARAMKEKLKN----KRIGIIISGGNVDIERAHFLSQ-----
               . **.*.:**.*.:**.*.:**.*.:**.*.:**.*.:**.*.:**.*.:**.*.:

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Figure 2.5 The alignment of eukaryotic serine racemase proteins. The CLUSTALW program with Biology Workbench at <http://workbench.sdsc.edu> was used to perform the alignment.

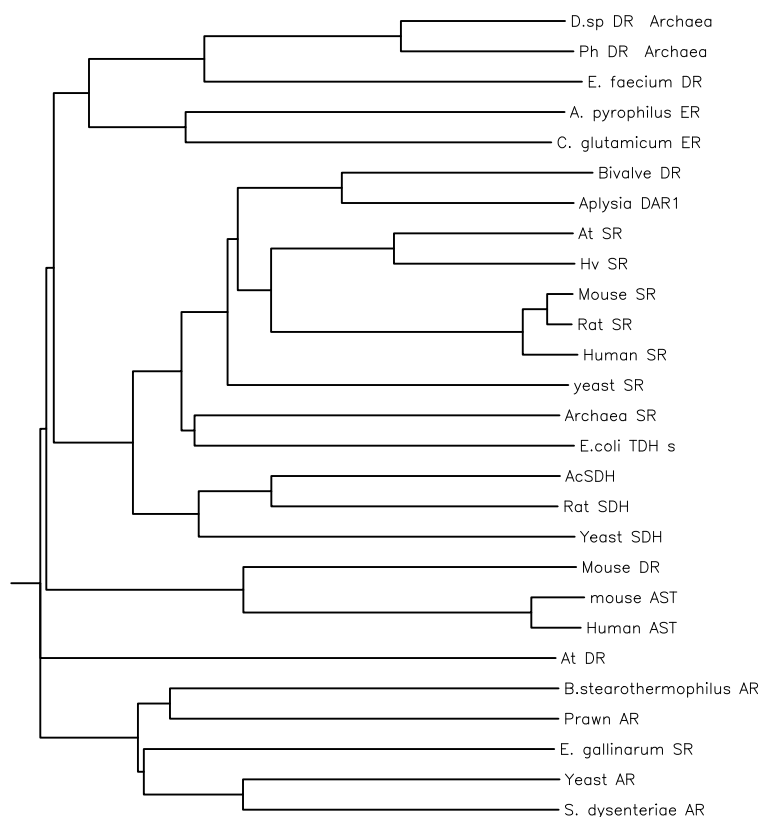


Figure 2.6 Twenty six characterized enzyme sequences were aligned using Clustal W program (<http://seqtool.sdsc.edu>) and the resulting alignments were displayed as inferring evolutionary tree by Phylip' Drawgram. TDH: threonine dehydratase; SDH: serine dehydratase; AST: aspartate aminotransferase; SR: serine racemase; DR: aspartate racemase; AR: alanine racemase; ER: glutamate racemase; DAR1: d-amino acid racemase 1; D.sp DR (dbj_BAA12209.1); Ph DR (NP_142620.1); *E. faecium* DR (ZP_05666819); *A. pyrophilus* ER (AAF25672.1). *C. glutamicum* ER (NP_601711.1); bivalve DR(BAE78960.1); DAR1(*A. californica* D-amino acid racemase 1, AC_HM776055); At SR (dbj_BAE72067.1); Hv SR (dbj_BAF63026.1); mouse SR(NP_001156783); rat SR(NP_942052); human SR(NP_068766); yeast SR (GI:71041740); Archaea SR (dbj_BAE54303.1); *E. coli* biosynthetic TDH (NP_418220); AcSDH (gb_AAU05774.1); rat SDH(NP_446414); yeast SDH (gb_AAA35040.1); mouse DR (30); mouse AST(AAA37263); human AST (S29028);At DR (NP_200593.2); *B. stearothermophilus* AR (P10724.2); prawn AR (dbj_BAH22617.1); *E. gallinarum* (AAD22403.1); yeast AR (O59828); *S. dysenteriae* AR (BAB71770.1).

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CHAPTER III

DISCOVERY AND DESCRIPTION OF D-AMINO ACID RACEMASE 1 (DAR1) FROM APLYSIA CALIFORNICA CENTRAL NERVOUS SYSTEM

Notes and Acknowledgements

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3.1 Introduction

D-serine (D-Ser) and D-aspartate (D-Asp) are endogenous molecules with known or putative cell-to-cell signaling activities in the CNS. D-Ser is predominantly found in forebrain structures (1), and acts as a co-agonist of the NMDA receptor, regulating activity at its glycine modulation site (2)}. Converted from L-serine (L-Ser) by the pyridoxal 5'-phosphate (PLP)-dependent serine racemase (SerR) in astrocytes (3), D-Ser can be degraded by D-amino acid oxidase (DAO) (4) and acts as a novel glial neurotransmitter/neuromodulator (5, 6). Abnormal levels of SR and DAO have been observed in schizophrenia patients (7-9), suggesting a connection between D-Ser levels and brain function, including mental illness.

In contrast to D-Ser, D-Asp is widely distributed in animals. It has been found in the brain, retina, and endocrine and exocrine tissues of both vertebrates and invertebrates (10). D-Asp displays a temporal pattern during animal development, beginning with transient high levels between embryonic and early postnatal stages, and rapidly declining to trace levels in most tissues in young adults (4, 11). Interestingly however, as animals mature, D-Asp is found at increasing levels in endocrine tissues (12). The enzyme required for the biosynthesis of D-Asp

in the brain was not identified until recently, when the first mammalian aspartate racemase, DR, was cloned and characterized (13), although, how much this enzyme accounts for overall D-Asp synthesis is unclear. While the mechanism by which D-Asp forms remains under investigation, its catabolism is well understood and occurs through the enzyme D-aspartate oxidase (14, 15).

Efforts to understand D-Asp function are also ongoing. Indirect evidence points to hormonal roles (10), whereas other studies have shown its involvement in vision (16), embryonic development (17, 18), learning and memory (19), and neurogenesis (13). Several groups, including ours, have proposed that D-Asp can act as a neurotransmitter (12, 14, 20-22).. Further research is required to elucidate the physiological role of this intriguing signaling molecule.

Clearly, many questions remain about D-Asp formation, regulation and ultimately, its function; uncovering the answers will require having knowledge of enzymatic D-Asp biosynthesis. Although the D-Ser biosynthesis pathway was described in 1999 (3), as noted, DR, the first D-Asp racemase enzyme from the animal brain, was just recently reported (13). DR converts L-Asp to D-Asp and its knockdown reduces newborn neuron survival and dendritic arborization in adult mouse hippocampus. In invertebrates, significant quantities of D-Asp are also found in nervous and endocrine tissues, indicating a potentially conserved D-Asp signaling pathway among Metazoan. However, the origin of the D-Asp in these tissues has not been clarified, nor

has a DR-like enzyme been reported in *A. californica*. So far, the only invertebrate Asp racemase described, SbAspR, was isolated from the foot muscle of the bivalve *Scapharca broughtonii* (23). But it is unknown if the enzyme is also present in bivalve nervous and endocrine systems. In a prior study (24), we detected high levels of D-Asp (% D/L + D) in the F-, C- and G-clusters of the cerebral ganglion of the *A. californica* CNS, with the highest D-Asp content (85%) found in the insulin-producing F-cluster cells. We also observed enzyme activity in the cerebral ganglion that could transfer [¹⁴C] from L-Asp to D-Asp in radioisotope pulse-and-chase experiments (21).

In this report, we describe the cloning of an Asp racemase gene, *dar1*, from the *A. californica* CNS, the characterization of a DAR1 protein by enzyme assay, and examination of CNS enzyme localization. Racemase assays were performed using small-volume capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection, selected because the methodology is well suited for separating chiral amino acids from small-volume samples (25, 26). We characterized the enzyme's activity and show that DAR1 converts L-Asp to D-Asp, and vice versa, with a much smaller K_m for L-Asp substrate than for D-Asp substrate. The enzyme also exhibits Ser racemase activity with a similar K_m for both L-Ser and D-Ser substrates. The distribution of DAR1 in *A. californica* indicates it is found in tissues previously shown to have significant levels of D-Asp. Based on this dual racemase activity, the same tissues have been characterized for D-Ser, and both D-Ser and D-Asp are shown to co-localize.

3.2 Materials and Methods

3.2.1 Experimental Animals

Adult *Aplysia californica* (125–250 g) were purchased from the *Aplysia* Research Facility in Miami, FL. Animals were kept in an aquarium of artificial sea water (460 mM NaCl, 10 mM KCl, 10 mM CaCl₂, 22 mM MgCl₂, 6 mM MgSO₄ and 10 mM HEPES, pH 7.8) with constant aeration at 14–15 °C and were used within two weeks of arrival. For dissection, animals were anesthetized with an intraperitoneal injection of MgCl₂ solution (390 mM) at 30–50 % of body weight (v/w), and the interconnected CNS ganglia removed with surgical tools. The ganglia were either immediately processed for RNA isolation, total protein extraction or amino acid extraction; or treated with 1% protease Type IX (Sigma-Aldrich) in artificial sea water containing 100 units/ml penicillin G, 100 µg/ml streptomycin, and 100 µg/ml gentamicin for 1 h at 34 °C to aid in the removal of connective tissues prior to CNS immunohistochemistry staining.

3.2.2 cDNA Cloning of the *dar1* Gene

The RNA preparation and DNA cloning reagents were purchased from Invitrogen unless otherwise specified. Chemicals were purchased from Sigma-Aldrich and the DNA oligos from IDT and the W.M. Keck Center for Comparative and Functional Genomics of the University of Illinois at Urbana-Champaign. *A. californica* genomic searches for potential amino acid racemase genes were conducted at the NCBI website (<http://www.ncbi.nlm.nih.gov>) using the SbAspR protein sequence (GeneBank accession:

BAE78960) as the search query. The target gene found from the BLAST search was named *dar1*. The cDNA sequence of the *dar1* was cloned from *A. californica* CNS ganglion poly(A)⁺ RNA templates prepared by TRIzol Reagent and Dynabeads by conventional RT-PCR, 5' RACE and 3' RACE cloning techniques using the GeneRacerTM SuperScript III module kit and following product instructions. Gene specific primers (GSPs) for RT and PCR reactions were created from the *dar1* gene and high fidelity DNA polymerases were used for the PCR reactions. PCR products of interest were cloned into pCR4.0-TOPO vector, transformed into TOP10 cells and sequenced. Three independent clones were obtained which covered the complete cDNA sequence of the *dar1* gene. Clone 3 contained almost the complete *dar1* protein coding sequence except for the last 15 nucleotides (nt). Clone 23 was produced by 5' RACE and contained the 5' UTR and a partial coding sequence overlapping with the 5' end of clone 3 by 882 nt. Clone 108 was produced by 3' RACE and contained the 3' UTR and a partial protein coding sequence overlapping the 3' end of clone 3 by 282 nt. From the sequence information obtained from these three clones, two GSP primers were designed and used to clone the entire *dar1* ORF by standard RT-PCR from the *Aplysia* CNS ganglion poly (A)⁺ with Platinum *Pfx* DNA polymerase. The final ORF clone was named T90.

The primers used for the RT and PCR reactions include the following. For clone 3 cloning: GeneRacerTM Oligo dT for RT; GSP 5'-GTGACACACTCCAGTGGGAAC-3' (GSP forward) and 5'-CTCAATGTCCAGATTCCCACC-3' (GSP reverse) with *pfu* Turbo DNA polymerase (Agilent Technologies) and the following thermal conditions: 94 °C for 2 min for 1 cycle;

94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min for 30 cycles; 72 °C for 10 min for 1 cycle. For clone 23 cloning, GeneRacer kit (Invitrogen) was used using RNA ligase-mediated 5' RACE. *Aplysia* CNS mRNA was first dephosphorylated with Calf Intestinal Phosphatase and then treated with Tobacco Acid Pyrophosphatase followed by ligation with GeneRacer RNA Oligo to the 5' end of the mRNA. The decapped and ligated mRNA was reverse transcribed with GSP reverse primer 5'-CTCAATGTCCAGATTCCCACC-3' and SuperscriptTM III transcriptase at 55 °C for 60 min. The first strand cDNA produced from the RT was amplified by PCR with GSP reverse primer 5'-AGCAGCGGCAACCGACGCCCC-3' and commercial GeneRacerTM 5' Primer 5'-CGACTGGAGCACGAGGACACTGA-3' as forward primer and Platinum[®] *pfx* DNA polymerase. The PCR thermal conditions used were: 94 °C for 5 min for 1 cycle; 94 °C for 30 sec, 69 °C for 30 sec, 68 °C for 1.5 min for 35 cycles; 68 °C for 10 min for 1 cycle. For clone 108 (3' UTR clone): DNase treated mRNA was used as template of RT reaction with GeneRacerTM Oligo dT and SuperscriptTM III enzyme, 55 °C for 60 min; for primary PCR, 5'-ATGGCAGCTTCGTGTGGAGTAACTT-3' (GSP forward) and GeneRacerTM 3' Primer (reverse), and Platinum[®] *Taq* DNA polymerase High Fidelity were used with the following touchdown and hot start PCR thermal profile: 94 °C for 3 min (add 0.5 µl enzyme to each reaction after 1 min at 94 °C) for 1 cycle; 94 °C for 30 sec, 72 °C 90 sec for 5 cycles; 94 °C for 30 sec, 70 °C for 90 sec for 5 cycles, 94 °C for 30 sec, 65 °C for 30 sec, 68 °C for 90 sec for 25 cycles; 68 °C for 10 sec for 1 cycle. Nested PCR was carried with 5'-CCGCCCCAGTACCTGGACACC-3' (GSP forward) and GeneRacerTM 3' Nested Primer (reverse), and Platinum[®] *Taq* DNA polymerase High Fidelity

with the following PCR thermal profile: 94 °C for 3 min for 1 cycle; 94 °C for 30 sec, 65 °C for 30 sec and 68 °C for 1 min for 25 cycles; 68 °C for 10 min. For T90 cloning: GeneRacerTM Oligo dT for RT; forward GSP primer 5'-GGCATATGGCAGCTTCGTGTGGA-3' (the NdeI site is underlined) and reverse GSP primer 5'-CCCTCGAGTCAAAAAGGCAAATTCTCAATGTC-3' (the XhoI site is underlined) for open reading frame full-length PCR with Platinum *pfx* DNA polymerase with the following PCR thermal profile: 94 °C for 3 min (hot started) for 1 cycle; 94 °C for 30 sec, 56 °C for 30 sec, 68 °C for 1 min for 39 cycles; 68 °C for 10 min.

3.2.3 Construction of the *dar1* Protein Expression Vector

The clone T90 plasmid and pET15b bacterial expression vector (EMD Chemicals) were double digested with NdeI and XhoI restriction enzymes (New England BioLabs, Inc.). The *dar1* fragment and cleaved vector were gel-purified, ligated and transformed into TOP10 cells. Transformants were mini-screened and sequenced. A sequence-confirmed clone was transformed into BL21 (DE3) bacterial cells (EMD Chemicals) to generate the bacterial clone 15bT90-31.

3.2.4 DAR1 Expression and Purification

Protein expression and purification reagents were purchased from EMD4Biosciences (Gibbstown, NJ) unless specified otherwise. Chemicals were purchased from Sigma-Aldrich

(St. Louis, MO). 15bT90-31 bacteria glycerol stock was inoculated in 2 ml LB supplement with ampicillin (100 µg/ml) and grown overnight. The cells were spun down and re-grown in 250 ml fresh LB with ampicillin at 37 °C for 3 h or until the O.D. 600 reached 0.4–0.5. The culture was then cooled down quickly on ice and then transferred to a temperature controlled shaker. The protein expression of *dar1* gene was induced by addition of 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture was continued at 17 °C for 16 h. His.tag protein purification from the bacterial cells was performed by following the BugBuster Mix lysis and nickel-nitrilotriacetic acid (NiNTA) purification product instructions with some modifications. Bacterial cells were harvested from overnight culture by centrifugation at 10,000 x g for 10 min at 4°C. The cell pellet was lysed with 10 ml of BugBuster Master Mix supplemented with 2 mM DTT and protease inhibitor cocktail following product instruction. The cell lysate was cleared by centrifugation at 16,000 x g for 20 min at 4 °C. The supernatant was mixed with 2 ml NiNTA resin in 10 ml NiNTA Bind Buffer (50 mM NaPO₄, 100 mM NaCl, 1 mM imidazole, 0.5 mM tris-3-carboxyethanol phosphine, or TCEP), pH 8.0, supplemented with 2 mM DTT and protease inhibitor cocktail (Novagen) and incubated with rocking at 4 °C for 1 h. The resin was then packed into a 24 ml Econo column (Bio-Rad Laboratories) by gravity at 4 °C and column purification was performed at 4 °C cold room. The column was washed with 40 ml NiNTA Wash Buffer (Bind Buffer with 20 mM imidazole) and protein was eluted with 7 ml NiNTA Elution Buffer (Bind Buffer with 250 mM Imidazole) into a tube with 5 ml Elution Buffer. The eluted protein was concentrated down to 2 ml with Amicon Ultra Centrifugal filter (Thermo Fisher Scientific) and subjected to

gel filtration chromatography with a HiPrep Sephacryl 200 HR 16/60 column (GE Healthcare Life Sciences) using 50 mM Tris-HCl buffer, pH 8.0 with 2 mM DTT at 1 ml/min flow rate and collected protein peaks at 1 ml fractions and tested for enzyme activity. The active fractions were pooled and buffer exchanged to enzyme storage buffer consisted of 50 mM NaPO₄, 150 mM NaCl, pH 8.0, 20% glycerol and 1 mM DTT. Protein concentration was determined with BCA assay kit (Thermo Fisher Scientific). Protein molecular size was determined with the gel filtration column described above and calibrated with Low Molecular Weight Standard kit (GE Healthcare Life Science) with the purification buffer and calculated based on peak retention time following product instructions. Protein molecular weight and purity were estimated by SDS-PAGE with 12% Tris-glycine gel (Invitrogen) with Coomassie staining. Enzyme activity was determined by serine racemase assay as described below.

3.2.5 *Aplysia* Tissue Protein Extraction and Western Blot

Four adult animals were anesthetized and tissues (CNS, ganglion, liver, buccal muscle, ovotestis, atrial gland) were dissected and pooled. CNS ganglia were desheathed to remove the fibrous connective tissues, as described above, before protein extraction. The desheathed CNS and other tissues were quickly frozen in liquid nitrogen and then homogenized with TissueRuptor in Qproteome protein preparation lysis buffer (QIAGEN). Total protein was extracted from the homogenized lysates by following the Qproteome kit instructions. Protein concentration was determined by BCA assay. Proteins were boiled in 1X Laemmli buffer containing 10 mM β -mercaptoethanol and loaded to 12% Tris-glycine gel and subjected to

reducing SDS-PAGE. Resolved proteins were transferred to HybondTM -P (GE Healthcare) PVDF membrane, blocked with 5% non-fat Carnation milk (NestléUSA) in Tris-buffered saline with Tween (50 mM Tris, 150 mM NaCl, pH 7.6, 0.05% Tween 20), stained with anti-DAR1 rabbit serum at 1: 30,000 and goat anti-rabbit HRP secondary antibody (Jackson ImmunoResearch) at 1: 20, 000. Antibody-treated membrane was reacted with ImmobilonTM Western chemiluminescent HRP substrate (Millipore) and exposed to Amersham HyperfilmTM (GE Healthcare Life Sciences).

3.2.6 CE-LIF Instrumentation and Sample Analysis

Separations were performed using an automated P/ACE MDQ CE system equipped with LIF detection (Beckman Coulter). The CE system was coupled to an external diode laser 56ICS426 (Melles Griot) emitting 440 ± 8 nm, with the coupling performed via a fiber optic cable (OZ Optics). The laser power was adjusted to 3 mW at the output terminus of the optic cable to avoid damage to the photomultiplier tube (PMT). A bandpass filter of 490 ± 15 nm (Omega Optical), located prior to the PMT, selects the appropriate fluorescence emission band for detection. For separations, uncoated fused-silica capillaries (Polymicro Technologies) were used. Capillaries were injected with 0.1 M NaOH at a pressure of 30 psi for 1 min and allowed to be primed in NaOH for 25 min before their initial use. Samples were introduced into the capillaries by pressure injection of 0.5 psi for 5 sec. The separation buffer for the Asp and glutamate (Glu) enantiomers contained 200 mM borate, pH 9.5, with 60 mM sodium deoxycholate (SDC) and 40 mM β -cyclodextrin (β -CD) (27). The Ser enantiomer separation

buffer contained 75 mM borate, pH 10.5, with 50 mM SDS and 10 mM γ -cyclodextrin (28). For chiral alanine (Ala) separation, the inlet buffer consisted of 25 mM phosphate, pH 2.2, containing 4% (w/v) sulfated β -CD, and the outlet buffer of 50 mM phosphate, pH 2.2 (29). For Asp, Glu, and Ser chiral separations, capillaries of 75 μ m inner diameter (ID) and 360 μ m outer diameter (OD) were used. Capillary total length was 80 cm with an effective length of 70 cm to the detection window. Separations were carried out by applying 27 kV of normal polarity. Between two runs, the capillaries were rinsed with methanol (30 psi, 0.5 min), 0.1 M NaOH (30 psi, 1 min), and separation buffer (30 psi, 1 min). For Ala enantiomer separations, capillaries of 50 μ m ID and 360 μ m OD were used with 60 cm total length and 50 cm effective length. A reverse polarity of 20 kV was applied for separations. After each run, the capillaries were rinsed first with methanol (30 psi, 0.5 min), followed by the inlet separation buffer (30 psi, 1 min). CE-LIF results were displayed as electropherograms, which show analyte peak fluorescence intensities against analyte migration time from the capillary injection site to detection window. For quantitative analysis, the peak areas of amino acid analytes and internal control glycine from the CE electropherograms were obtained using Origin 8 software (Origin Lab Corporation). In order to obtain analyte concentrations, analyte peak areas were divided by glycine peak areas to generate normalized analyte peak area values, and converted to concentrations using working curves generated with known concentrations under the same experimental conditions.

3.2.7 DAR1 Racemase Assay (non-quantitative assay)

Chemicals and reagents for the enzyme assays were of the highest grade available and purchased from Sigma-Aldrich unless specified otherwise. Naphthalene-2,3-dicarboxaldehyde (NDA) was purchased from Invitrogen. Water was deionized and purified using a Barnstead E-PURE purification system (ThermoFisher Scientific) and sterilized by passing through 0.22 μm filters. Procedures were performed at room temperature unless otherwise noted. The racemase assays contained a cofactor mix composed of 20 μM PLP, 4 mM ATP, 2 mM DTT and 2 mM MgCl_2 in Tris-HCl buffer of various concentrations and pH, depending on the specific experiment. To perform each assay, L/D-Asp, L/D-Ser, L-Glu or L-Ala (2.5–200 mM) substrates were incubated with 1–10 μg of purified DAR1 in 100–150 μl Tris-HCl buffer (25–50 mM, pH 7.0–8.5) and cofactor mix at 30 $^{\circ}\text{C}$ for 2–4 h. Negative controls contained heat-inactivated enzyme or no substrates. At the end of the incubation period, glycine (3–5 mM) was added to each reaction and used as an internal control for subsequent sample treatments and analyses. Enzyme was removed by either TCA precipitation or the alternative filtration method described below, before amino acid derivatization for CE analysis. The TCA precipitation method was used for the Ser and Asp assays. To remove the enzyme, 5% cold TCA was added to the reaction samples and incubated for 10 min on ice followed by centrifugation at $10,000 \times g$ for 10 min. TCA was then removed from the samples by two extractions with equal volume of water/saturated ether. Enzyme can also be removed from an assay sample by centrifugation in a Pierce[®] Concentrator 7ml/9K molecular-weight cutoff (ThermoFisher Scientific) for 30 min to collect protein-free filtrate. Protein-free samples were diluted in 50 mM borate buffer, pH 9.4, to appropriate volumes so that amino acids were

derivatized with an 8× excess of NDA and 20× excess of potassium cyanide (KCN) in 50 mM borate buffer, pH 9.4, for 45 min. The reaction was stopped by diluting 1:10 with 50 mM borate buffer, pH 8.5, and immediately frozen on dry ice. Samples were then subjected to CE-LIF analysis within 24 h.

3.2.8 Enzyme Kinetics Assay (quantitative assay)

A series of concentrations (2.5–80 mM) of Ser or Asp substrates were incubated with 1–2 µg of purified enzyme in 100 µl reactions in 50 mM Tris-HCl buffer, pH 8.5, for Ser reactions, or pH 8.0 for Asp reactions, containing cofactor mix at 30 °C for 70–150 min. At the end of the incubations, 5 mM glycine was added to each reaction as an internal control. In addition, L-Ala was added to the Ser samples and L-Glu was added to the Asp samples so that the total amino acid concentrations in all reactions were equal to the highest substrate concentrations. The enzymes were immediately removed by centrifugal filtration as described above. The protein-free samples were then diluted to appropriate concentrations with 50 mM borate buffer, pH 9.4, so that 800 µM of the total amino acids were derivatized with 6.4 mM NDA and 16 mM KCN in a 10 µl reaction for 60 min. The reactions were stopped by 1:10 dilution with 50 mM borate buffer, pH 8.5, and quickly frozen on dry ice until CE-LIF analysis. The quantity of amino acid products was calculated from the linear function produced by a standard amino acid calibration curve by co-assaying standard amino acids without enzyme with samples containing enzyme. The enzyme catalysis velocity is defined as a µmole product formed by 1 mg of enzyme during a 1 h reaction time at 30 °C under assay-described buffer

conditions. The inverse enzyme velocities were plotted against inverse substrate concentrations and fitted to a linear model using Microsoft Excel to generate Lineweaver-Burk Plots. The K_m and V_{max} values were calculated from the inverse X- and Y- intercepts of the plots.

3.2.9 *Aplysia* CNS Whole-mount Immunohistochemistry

A rabbit polyclonal antibody against purified full-length DAR1 was generated by the Immunological Resource Center, University of Illinois at Urbana-Champaign. A New Zealand White female rabbit of 3-6 month old was immunized via s.c. with 500 μ l - 1 ml antigen emulsion composed of DAR1 and adjuvant at 1:1 ratio (v/v) four times, once every three weeks and then bled out by cardio-puncture method. For primary immunization, 500 μ g of antigen and Titermax (SIGMA) adjuvant was mixed by sonication to make antigen emulsion; and 250 μ g DAR1 and Incomplete Freund's Adjuvant emulsion was used for all subsequent immunizations. All animal work was performed in compliance with federal, state and university guidelines for animal care and usage. Normal goat serum and rhodamine Red-X-conjugated goat anti-rabbit IgG (H+L) secondary antibody were purchased from Jackson ImmunoResearch (West Grove, PA). Paraformaldehyde was purchased from Electron Microscopy (Sciences Hatfield, PA). The periesophageal ring and abdominal ganglia without connective tissue (or desheathed) were stretched onto Sylgard in 35 mm Petri dishes and fixed with paraformaldehyde as described by Llewellyn-Smith et al. (30). The tissues were rinsed with PBS to remove the fixative, blocked overnight with 10% normal goat serum, and then

incubated with anti DAR1 rabbit antibody (1:1000 diluted) for 7 days. The samples were washed with 10 mM phosphate buffer, pH 7.5, containing 154 mM NaCl, 1% BSA, 0.25 mM thimerosal and 2% Triton X-100 for 3 days, incubated with the secondary antibody (1:500 dilution) for 3 d in the dark, and washed with the phosphate buffer without Triton X-100 for 3 days. Control samples were stained with either primary antibody or secondary antibody alone or pre-immune serum plus secondary antibody. The post-staining preparation for light microscopy visualization was performed as described by Fujisawa et al. (31). Basically, the samples were dehydrated for 30 min in each of the following ethanol solutions by order: 30 %, 60 %, 90 %, 100% and 100 %. The samples were then cleared by soaking in 98 % methyl salicitate for 15 min and then incubated at 4 °C for overnight. The samples were mounted on glass slides and kept in the dark until photographed within a week with a Zeiss Axiovert 200M inverted research-grade microscope equipped with a Roper Scientific Cascade 512B EMCCD camera and Zeiss Axiovision software. The camera obtained black/white and color fluorescence images with appropriate filters; the software was used to perform Z-stacking, time-lapse and tiling. The rhodamine images were obtained via 560 nm excitation and 620 nm emission.

3.2.10 Detection of D-Asp and D-Ser in *Aplysia* CNS Ganglion Clusters

Aplysia californica cerebral ganglion clusters were dissected from anesthetized animals and immediately placed into 100–200 µl of 0.1 N HCl. The cells were homogenized manually with a glass homogenizer followed by sonication for 15 min in a water-bathed sonicator. The

materials were then centrifuged at $10,000 \times g$ for 10 min at 4 °C and the supernatants dried down in a microcentrifuge under vacuum. The dried materials were reconstituted with 10 μ l of 100 mM borate buffer, pH 9.4. Samples (0.5 μ l) were then derivatized with 1 μ l of 20 mM NDA and 1 μ l of 20 mM KCN for 1 h. The derivatized samples were diluted 5–20 fold with 75 mM borate buffer, pH 10.5, for Ser separation, or 200 mM borate buffer, pH 9.5, for Asp separation. The diluted samples were injected into capillary by pressure at 0.5 psi for 5 sec and separated under 27 kV with normal polarity.

3.3 Results

3.3.1 Cloning of *dar1* Full-length cDNA

In order to locate an aspartate racemase in the *A. californica* nervous system, we searched the online *A. californica* genome database and discovered a candidate gene (gi-225479051), naming it *dar1*. We cloned the full-length cDNA of the *dar1* gene, including the protein coding sequence (deposited in GenBank HM776055), and the 5' UTR and 3' UTR from poly(A)⁺ RNA, by using conventional RT-PCR, 5' RACE and 3' RACE cloning techniques. The cDNA sequence and protein translation of DAR1 are shown in Fig. 3.1. The cDNA includes a 20 nt 5' UTR, 978 nt coding sequence and 241 nt 3' UTR. The 3' UTR contains a polyA tail of 23 adenines and two overlapping AAATAA polyadenylation signals. In total, the cDNA contains 1220 nt and codes for a 325 amino acid-long protein. At the protein level, DAR1 shares a 55% sequence identity to the bivalve aspartate racemase, and more than 40% to human, rat and mouse brain serine racemases. A multiple sequence alignment of these five

related proteins is shown in Fig. 3.2. The proteins have similar lengths, ranging from 325 to 340 amino acids, and share multiple conserved regions, including a putative PLP-binding motif near the N-terminus around the DAR1 PLP-binding Lys57 (32) and the DAR1 PLP-binding stabilizing clusters, 80Ser-Ser-Gly-Asn-His and 183Ser-Gly-Gly-Gly (33).

3.3.2 DAR1 Expression and Purification

We cloned the *dar1* ORF into a microbial expression vector, over-expressed the gene as a soluble recombinant protein with an N-terminal 6xHis.tag in *E. coli*, and purified it for functional studies. The recombinant protein was only expressed under IPTG induction and purified from bacterial cell lysate to near homogeneity using NiNTA affinity purification and gel filtration chromatography Fig. 3.3 The recombinant DAR1 exhibited an electrophoresis mobility of 37 kDa in a reducing SDS-gel—a result consistent with the predicted molecular weight of the recombinant protein, but it had a molecular mass of 57 kDa as determined by a sizing column. The considerably larger molecular mass than molecular weight indicates that DAR1 molecules form homodimers in solution. Dimerization seems to be essential for DAR1 activity because the protein fraction of 30–35 kDa off the sizing column had little enzyme activity (data not shown).

3.3.3 DAR1 Expression in *A. californica* Tissues

We raised a rabbit polyclonal antibody against purified recombinant DAR1 and examined *dar1* expression in *A. californica* CNS neurons, and liver, buccal muscle, ovotestis and atrial

gland tissues by Western blot. As shown in Fig. 3.4, *dar1* was expressed as a ~36 kDa protein (predicted molecular weight is 35.4 kDa) in the CNS ganglion lysate. The protein was also strongly expressed in atrial gland- an exocrine tissue that secretes egg laying peptides, moderately in liver, and minimally in buccal muscle and ovotestis when an equal amount of total protein was loaded for each tissue sample. However, ovotestis showed a larger band with moderate signal which might be from a different transcript.

3.3.4 DAR1 Racemase Activity and Characterization

I examined DAR1 PLP-dependent racemase activity towards L/D-Asp, L/D-Ser, L-Glu and L-Ala substrates, and observed enzyme behaviors under different temperatures and buffer pH levels, and the effects of ATP/MgCl₂ on the enzyme activity. These measurements were accomplished with CE-LIF, an approach well suited for chiral amino acid separation and detection. Our assay results demonstrated that DAR1 was active towards both Asp and Ser substrates and converted these chiral enantiomers in both directions as shown in Fig. 3.5. However, DAR1 had no detectable activity when 10 µg of purified enzyme was incubated with 100 mM L-Glu or 100 mM L-Ala (D-Glu and D-Ala were not tested) in a 150 µl reaction containing 50 mM Tris-HCl, pH 8.0, with cofactors at 30 °C for 3 h, a condition where Asp and Ser conversion were easily observed.

DAR1, like other PLP-dependent racemases, requires the cofactor PLP for its activity. Purified enzyme appeared greenish yellow and absorbed light at 420 nm, a characteristic of

PLP-bound enzyme (33). The absorption peak disappeared after the enzyme was incubated for 10 min at room temperature with 2 μ M aminooxyacetic acid (AOAA), a PLP-inactivating reagent (data not shown). The enzyme did not lose color after a 48 h dialysis, indicating a high affinity between the enzyme and PLP. AOAA inhibited the conversion by DAR1 of D-Ser and D-Asp from their corresponding enantiomer (Fig. 3.6A). The thermal stability profile of DAR1 is shown in Fig. 3.6B. The enzyme activity increased between 14–45 °C and started to decrease at 50 °C for both the Ser and Asp conversions. We examined the modulating effects of ATP and MgCl₂ on DAR1 racemase activity (Fig. 3.6 continued). MgCl₂ (2 mM) had only a small effect on enzyme racemase activity but ATP (4 mM) significantly increased enzyme activity. When both effectors were present, the enzyme showed the largest activity for the D-Asp and D-Ser conversions. The ATP and MgCl₂ concentrations were not optimized for DAR1; we chose the concentrations based on published research on Ser and Asp racemases (34, 35). Finally, Fig. 3.6 continued showed the pH-dependent DAR1 L-Asp racemase activity (A) and L-Ser racemase activity (B). The enzyme showed little racemase activity below pH 6.5 (data not shown) and an increasing activity between pH 7.0–8.5 in 50 mM Tris-HCl buffer.

3.3.5 Enzyme Kinetics

The DAR1 Asp and Ser racemase kinetics linear curves are shown as Lineweaver-Burk Plots in Fig. 3.7. Analyzing the linear fits, the K_m (mM) was determined to be 8 ± 2 (SD) for L-Asp, 94 ± 30 for D-Asp, and 15 ± 4 and 16 ± 2 for L-Ser and D-Ser, respectively; the V_{max} (μ mole

hr⁻¹ mg⁻¹) for the same substrate order was determined to be 3.3 ± 0.8 , 7.9 ± 1 , 11 ± 1.6 and 15 ± 3 , respectively.

3.3.6 Co-localization of DAR1 and D-amino Acids in the *A. californica* CNS

We raised a rabbit polyclonal antibody against DAR1 and used it to perform whole-mount (versus section) immunohistochemistry with *A. californica* CNS material. We stained the isolated CNS, including the buccal, pleural, pedal, abdominal and cerebral ganglia, and their connective and peripherally extending nerves. The immunohistochemistry experiments revealed a highly localized distribution of DAR1 protein in the CNS (Fig. 3.8). The antibody exclusively stained F-, C-, G- and B-cluster cells located in the middle region of the fused cerebral ganglia. The staining was specific; controls without primary or secondary antibody produced only background signal (data not shown). The other ganglia showed only background staining, except for a group of 3–4 unidentifiable neurons in the vicinity of B1 and B2 landmark neurons in the buccal hemiganglia and a lone neuron in the abdominal hemiganglia.

While our prior research characterized D-Asp, we had not performed D-Ser separations; the optimum CE conditions are distinct for the two amino-acid pairs. Thus, we have re-measured both D/L-Asp and D/L-Ser within several CNS structures. As demonstrated as the CE-LIF electropherograms in Fig. 3.9, not only was a high level of D-Asp (more than L-Asp) once

again detected, a significant amount of D-Ser (less than L-Ser) was also found for the first time in the F/C-clusters.

3.4 Discussion

We have characterized a novel racemase enzyme from *A. californica*. Homology data suggests that the known mammalian and our new molluscan amino acid racemase share a conserved structural similarity. Intriguingly, although a similar enzyme has not been found in mammals, a distinct aspartate racemase (DR) was recently identified (13). DR has less than 20% identity to the other five racemase proteins, and it lacks the similar PLP binding and stabilizing sequences shared by other racemase groups. Based on our phylogenetic analysis, Ser racemases are closer to serine dehydratase (EC 4.3.1.17), while DR is closer to glutamate-oxalacetate transaminase enzyme (EC 2.6.1.1). It appears that DAR1 and DR are distinct Asp racemases from animal brains. Intriguingly, DAR1 and mammalian SerR are closely related and yet the mammalian DR is distinct. Are there other animal amino acid racemases that are still unknown? Given recent reports of high levels of D-Glu (36) and D-Ala (37) in rat brain, this appears a certainty. It will be interesting to determine their structures and how they fit within the growing Metazoan CNS amino acid racemase family.

After expressing and purifying DAR1, we tested its activity and ability for racemizing the amino acids that have previously been shown to be present at high levels in animal brains—Asp, Ser, Glu, and Ala (38, 39). The enzyme was active towards Ser and Asp enantiomers but

showed no activity towards Glu and Ala. To our best knowledge, DAR1 is the first characterized eukaryotic racemase that can catalyze the racemization of two substrates. The enzyme is PLP-dependent and appears to require dimerization for activity. Homodimers have been observed with mouse SerR and SbAspR (32, 34, 40). The recombinant DAR1 appears to be heat-tolerant under the assay conditions used. As with most protein enzymes, its activity increases with increasing temperatures. The enzyme shows a wide temperature range of activity, between 14 °C and 45 °C.

We examined ATP and MgCl₂ effects on DAR1 racemase activity and found that both reagents, especially ATP, are important for promoting enzyme racemase activity, and they act more effectively together than they do alone. It has been proposed that ATP and MgCl₂ exert allosteric regulatory effects on mouse SerR by lowering enzyme K_m (34). Although not examined, the same regulatory mechanisms might be at work for DAR1. Studies by other groups have also shown that other nucleotides and bivalent cations could regulate animal Asp and Ser racemase activities. As examples, besides Mg²⁺, Ca²⁺ and Mn²⁺ have been shown to promote mouse SerR activity (34) and AMP promoted SbAspR activity, but ATP reduced it (35). It is interesting that while ATP has a positive effect on DAR1 activity, the same nucleotide showed an inhibitory effect on SbAspR activity. The opposite effects of ATP on the two closely related mollusk aspartate racemases indicate that the enzymes may have different physiological functions. In addition, SbAspR might have a role in energy metabolism under anoxic conditions in bivalve muscle tissues (23).

The enzyme is more active under alkaline condition. Both Ser and Asp activities were low below pH 6.5, while increasing between pH 7.0 and pH 8.5 in 50 mM Tris-HCl. The pH profile is similar to that of a mouse SerR (3) and distinct from that reported for SbAspR (23), where the enzyme activity started to decrease above pH 8.5. We found that the optimal buffers were 25 mM Tris-HCl, pH 8.5, for L-Asp conversion and 50 mM Tris-HCl, pH 9.0, for L-Ser conversion (data not shown), suggesting that ion strength and pH can affect enzyme efficiency for a particular substrate. We chose 50 mM Tris-HCl, pH 8.5, for most of our assays in order to simplify the experimental procedures.

We determined the K_m and V_{max} for the four racemase reactions catalyzed by DAR1 as shown in Fig. 3.7. The kinetic data revealed the following. First, the enzyme favored L-Asp over D-Asp—the smaller the K_m , the higher affinity a substrate has for an enzyme—and the K_m for L-Asp substrate was about 10× smaller than that for D-Asp substrate. Although the enzyme had twice as large a V_{max} for L-Asp production, L-Asp is 10× more competitive than D-Asp for binding to the enzyme. Therefore, the enzyme was more efficient in producing D-Asp from L-Asp than vice versa. Differing from the Asp kinetics, Ser kinetics are comparable with both substrates. But the K_m values for both forms of Ser were still 4× larger than that for L-Asp. Based on the K_m values, the enzyme had the highest affinity towards L-Asp, followed by L-Ser/D-Ser, and then D-Asp. However, more studies are required to understand the physiological meaning of these kinetics parameters.

The selected presence and relatively sparse distribution of DAR1 within the *A. californica* CNS, specifically several cerebral ganglion clusters and isolated single neurons in the buccal and abdominal ganglia, suggests that the enzyme serves a specific function within these areas (and does not have a more general "cellular housekeeping" role). At this point, we have determined that DAR1 is capable of synthesizing D-Asp and it is localized to specific structures. Using CE-LIF we measured the presence of D-Ser and D-Asp in the cells containing the enzyme to determine if they co-localize. It is particularly telling that the enzyme was present in the F-, C- and G-clusters where high levels of D-Asp content were previously reported (21, 22), we now confirm the co-localization of D-Ser and D-Asp in these structures. The correlation of DAR1 and its products supports our determination that the enzyme is involved in the actual biosynthesis of D-Asp and D-Ser. Several years ago, we hypothesized that D-Asp was a neurotransmitter in the *A. californica* CNS (22). Since then, we have accumulated evidence of D-Asp release from and uptake by the cerebral ganglia. The finding of a D-Asp biosynthesizing enzyme in the cerebral ganglia substantiates this hypothesis.

3.5 Conclusions

We have isolated a novel racemase from *A. californica* neuronal tissues, characterized its biochemical features, and validated its ability to form D-Asp and D-Ser. DAR1 can catalyze racemization reactions between Asp and Ser enantiomers with differing kinetics. The enzyme

K_m and V_{max} values suggest a substrate preference order of L-Asp > L-Ser \geq D-Ser > D-Asp. DAR1 is the first Asp and Ser racemase discovered in an invertebrate brain and the first eukaryotic dual Asp/Ser racemase described. Using whole-mount CNS tissue staining with DAR1 antibody, we localized DAR1 to the central region of the cerebral ganglia where the F- and C-clusters are situated. These clusters contained a high level of D-Asp and a significant amount of D-Ser. The co-localization of the enzyme with the four amino acids demonstrates that DAR1 plays a role in the biosynthesis of D-Asp and/or D-Ser in the F/C-clusters. Future work will examine the roles of D-Asp and D-Ser in cell-to-cell communication and study the interactions between these two putative signaling molecules.

3.6 Figures

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-20  ggtcaaatagcaactaaacaATGGCAGCTTCGTGTGGAGTAACTTTCGTTCGATGTGCTC  39
      M A A S C G V T F V D V L
40  AAAGCACTGGAAAGGATTTACCGTTCATTACAAAACTCCTATCTTCACATCTGAACAA  99
      K A L E R I S P F I H K T P I F T S E Q
100  GCAAATAGGAAATGTGGACGACAACTGTTCTCAAATGCGAAAACTTTCAAAAGTCGGGT  159
      A N R K C G R Q L F F K C E N F Q K S G
160  TCATTTAAAGCCCGTGGAGCGTTGAATGCCGTTTGAAGTGTGAGCAAGTGAACCAAAC  219
      S F K A R G A L N A V L K C Q Q V K P N
220  GTCAACGGAGTGGTGACACACTCCAGTGGGAACCACGGCCAGGCGCTGGCATGGGCCGCC  279
      V N G V V T H S S G N H G Q A L A W A A
280  CAGCGAGCCAATCTCCCGTGTTCGTCGTCGTCACAGATGGCTCCTGATGTGAAGAAG  339
      Q R A N L P C C V V V P Q M A P D V K K
340  AATGCAATCCGAGGCTATGGAGCAGAGCTGTTGAATGTGGACCAAAGCCCAGTGACAGA  399
      N A I R G Y G A E L L E C G P K P S D R
400  AATGAGGCTTGTGACAAAGTACAGGACGATAGGAACCTTTGAGTTAATCCCCCTTATGAC  459
      N E A C D K V Q D D R N F E L I P P Y D
460  CACGTGGATGTGATTGCTGGACAGGGCACCATAGCTGTGGAGCTGTTGGAGCAAGTGCCT  519
      H V D V I A G Q G T I A V E L L E Q V P
520  TTCCTGGACGCTATCCTGGTCCCATCAGTGGAGGTGGGATGTCCTCGGGAATCTGTATC  579
      F L D A I L V P I S G G G M S S G I C I
580  GCTGCCAAGACCATCAAACCGGACATCAAATATTCATCGTGGCTCCTAAAGGGAAGCGA  639
      A A K T I K P D I K I F I V A P K G K R
640  CTGGAGGAATGTTTGAGAACAGGCAAGCGTCCTTGGGAAGGGCCGCCAGTACCTGGAC  699
      L E E C L R T G K R P W E G P P Q Y L D
700  ACCATTGCGACGGCATCCGGCTACAGCAGACGGGCTACATCACCACACCATACTCATG  759
      T I A D G I R L Q Q T G Y I T T P I L M
760  GAACTGGCCGAGAAAGACGTCTTTGAGATGAGTGATGAAGAAATTATTGAAGGAATGAAG  819
      E L A E K D V F E M S D E E I I E G M K
820  TTCAGCTTCGAGAGAATGAAGCTGGTCATTGAGACAGCGGCCGGGCGTCGGTTGCCGCT  879
      F S F E R M K L V I E T A A G A S V A A
880  GCTTCTCTGATCGACTACGGAAGATGGACCCGACTTAAAGAACGTTGGGGTCATTTTG  939
      A F S D R L R K M D P D L K N V G V I L
940  TGTGGTGGGAATCTGGACATTGAGAATTTGCCTTTTGAagagggtgaggagaggtcgag  999
      C G G N L D I E N L P F *
1000 gaaggggggtgaggggcaaagttgaggtgggatggtgaggaggggattaagatgaggagtg  1059
1060 tgagaagggatggttaacaaaaagcagcctcgtggtatccctttaacaagtaccaacag  1119
1120 aaaacagcaacaacaaaaatacaaatataataaaaacaaactaactaacgaagaaag  1179
1180 aaagaaagaacgagagaaaaa 1220

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Figure 3.1 Complete cDNA sequence and predicted protein sequence of *dar1* gene. 5' UTR: nt -20 to nt -1 in lower case letters; amino acid coding sequence: uppercase single letters; predicted amino acid sequence: single letter codes in uppercase; asterisk: stop codon TGA; 3' UTR: nt 979 to nt 1220 in lower case letters; polyadenylation signals: bolded lowercase letters (aataaa). Underlined sequence: predicted PLP-binding motif with a PLP-binding residue Lys56 in bold and shade. GenBank accession number for complete CDS: HM776055. Reprinted with permission from reference 41.

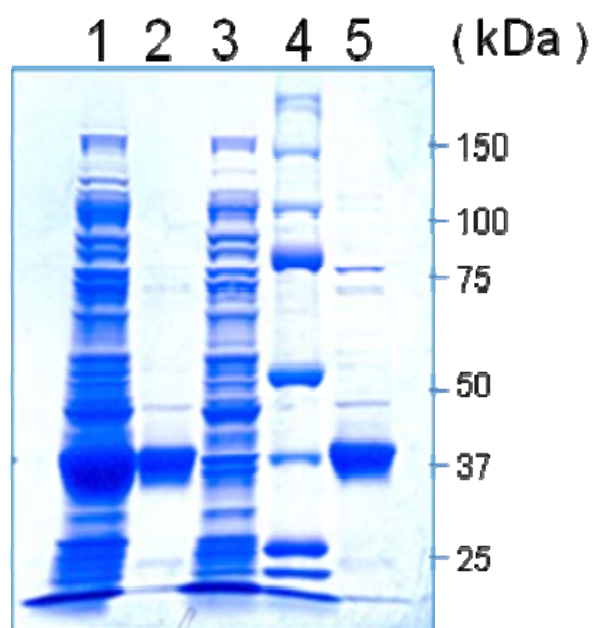


Figure 3.3 SDS-PAGE analyses of DAR1 expression and purification. Denatured and reduced cell lysates of bacterial cultures with IPTG (lane 1) or without IPTG (lane 3); 2.5 µg NiNTA purified protein (lane 5) and 2.5 µg gel filtration purified protein (lane 2); and 5 µg Precision Plus Protein standards (lane 4, Bio-Rad Laboratories, Hercules, CA) were loaded to 12% Tris-glycine gel. The SDS gel was stained with Coomassie blue. Predicated recombinant DAR1 molecular weight is 37.1 kDa. Reprinted with permission from reference 41.

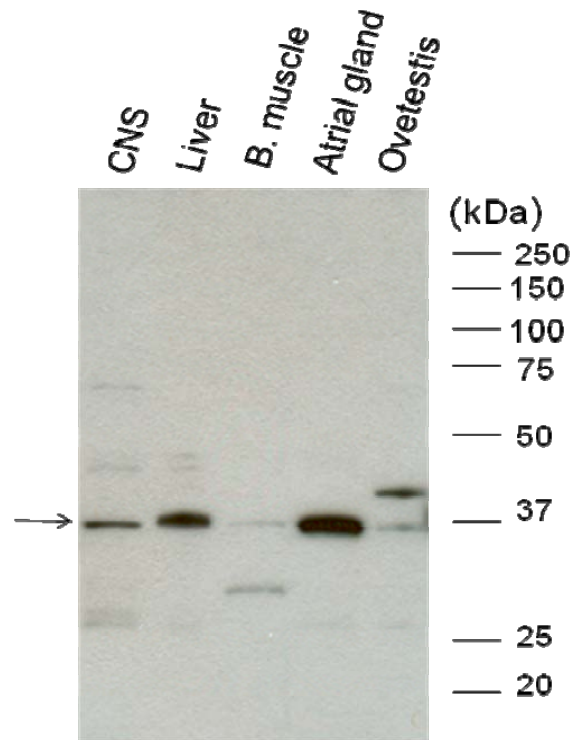


Figure 3.4 Western analysis of DAR1 expression in *Aplysia* tissues. Tissue protein lysates of CNS, liver, buccal muscle, atrial gland or ovetestis were subjected to reducing SDS-PAGE. Ten micrograms of each sample were co-run with Precision Plus Protein Standards in 12% Tris-glycine gel. Transfer blot was stained with rabbit anti-DAR1 serum (1: 30,000) and goat anti-rabbit HRP conjugate (1: 20,000). Film was exposed for 5 min. Predicted native DAR1 protein is 35.4 kDa. Reprinted with permission from reference 41.

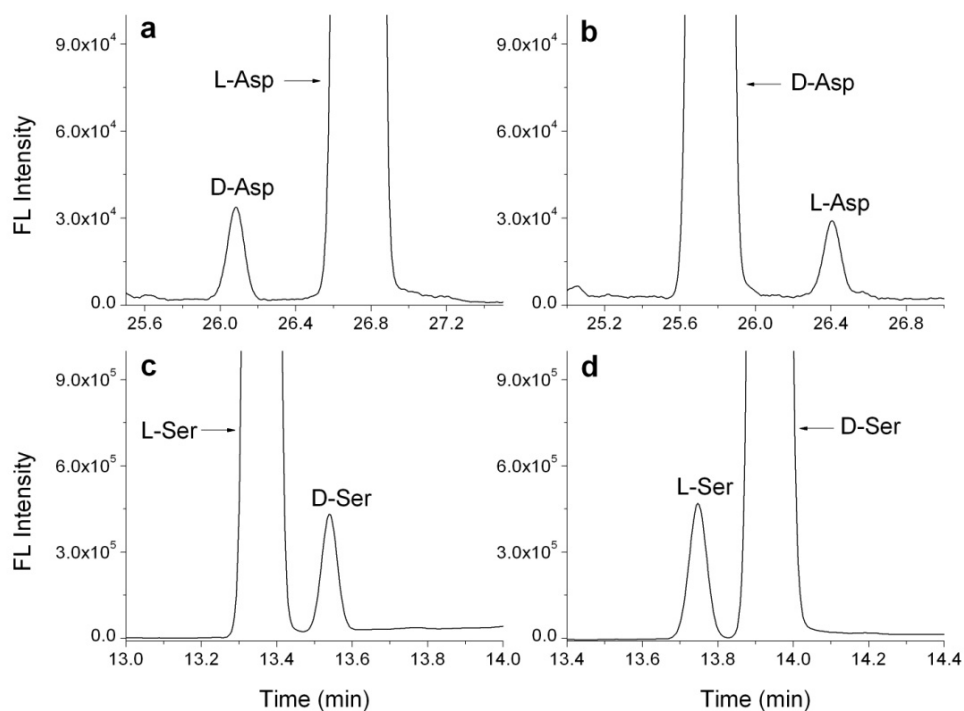


Figure 3.5 DAR1 Asp and Ser racemase activities. CE-LIF electrophoregrams of enzyme reactions with four substrates L-Asp (a), D-Asp (b), L-Ser (c), D-Ser (d). L-Asp/D-Asp (80 mM), or L-Ser/D-Ser (10 mM) was incubated with 1 μ g enzyme and cofactors in 150 μ l reaction buffer of 50 mM Tris-HCl, pH 8.0 (Asp reactions) or in 25 mM Tris-HCl, pH 9.0 (Ser reactions) at 30 $^{\circ}$ C for 2.5 h. Substrate starting concentration of 12 μ M L-Asp, 4 μ M D-Asp, 20 μ M L-Ser or 20 μ M L-Ser was injected into capillary. RFU: relative fluorescence units of analyte peaks. Migration time: time for analytes moving from capillary injection site to detection window. Reprinted with permission from reference 41.

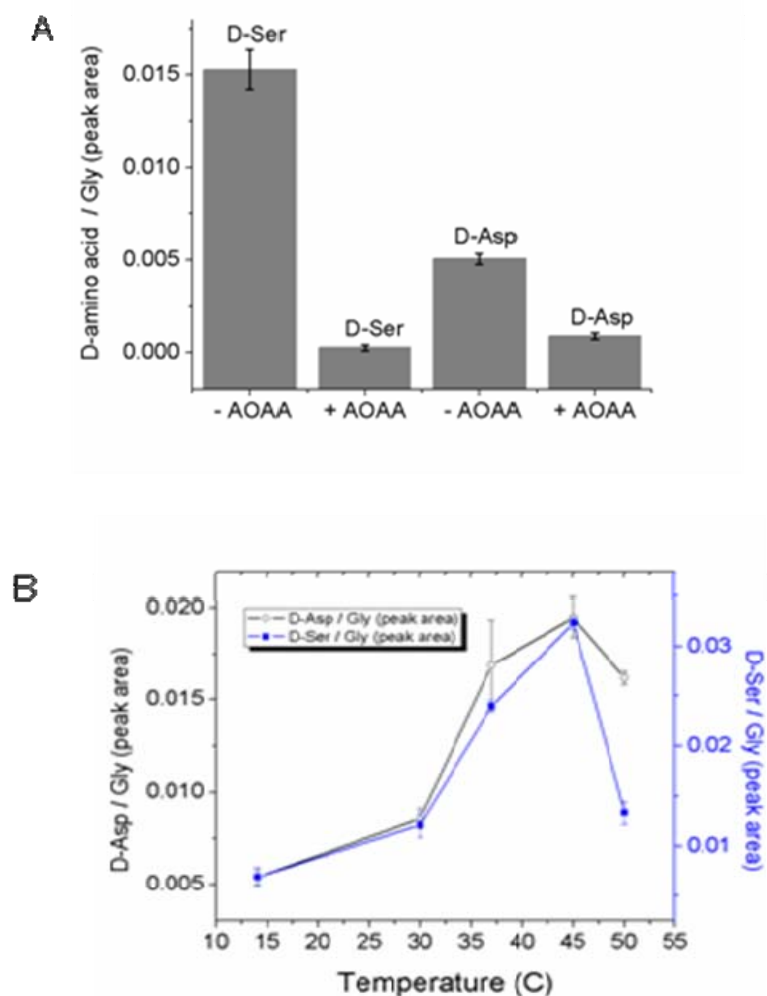


Figure 3. 6 DAR1 racemase activity characterizations. AOAA inhibition assay (A): 50 mM L-Asp or 20 mM L-Ser was incubated with 1 mM AOAA, 4 μ g DAR1 and cofactors in 150 μ l of reaction in 25 mM Tris-HCl, pH 8.5 at 30 $^{\circ}$ C for 2 h. Reaction concentration of 100 μ M L-Asp or 40 μ M L-Ser was injected into CE capillary. Thermo-stability of DAR1 racemase activity (B): 80 mM L-Asp or 50 mM L-Ser was incubated with 1 μ g DAR1 and cofactors in 100 μ l reaction in 50 mM Tris-HCl, pH 8.5 at 14 $^{\circ}$ C, 30 $^{\circ}$ C, 37 $^{\circ}$ C, 45 $^{\circ}$ C or 50 $^{\circ}$ C for 3 h (Asp) or 1 h (Ser). Reaction concentration of 100 μ M (L-Ser) or 160 μ M (L-Asp) was injected into capillary. Tris-HCl with pH7.0 (a, e), 7.5 (b,f), 8.0 (c,g) or 8.5(d, h) at 30 $^{\circ}$ C for 3 h. Reaction concentration of 1.77 μ M L-Ser or 17.7 μ M L-Asp was injected into capillary. Reprinted with permission from reference 41.

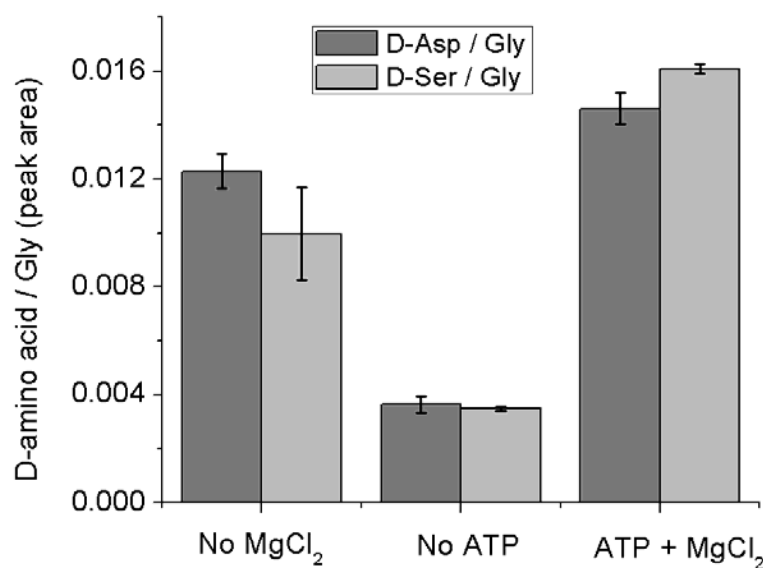


Figure 3.6 (cont.) Effects of ATP and MgCl₂ on DAR1 racemase activities. L-Ser (20 mM) or L-Asp (200 mM) was incubated with 2 µg DAR1, PLP and DTT and either 4 mM ATP or 2 mM MgCl₂ or both in a 100 µl reaction in 50 mM Tris-HCl, pH 8.5 at 30 °C for 1 h. Reaction concentration of 40 µM L-Ser or 100 µM L-Asp was injected into capillary. The data were adjusted by controls with heat-inactivated enzyme. Reprinted with permission from reference 41.

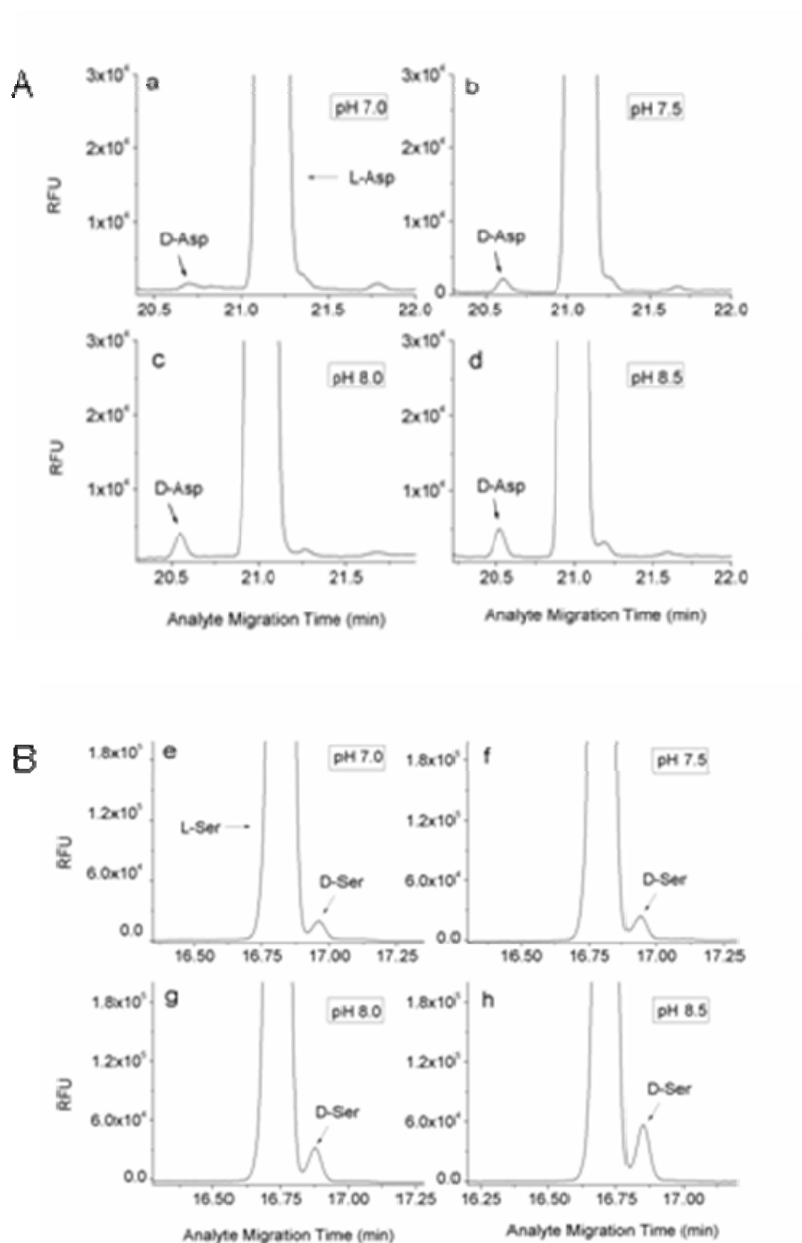


Figure 3.6 (cont.) pH-dependency of DAR1 racemase activity: CE-LIF electrophoregrams of DAR1 racemase reactions with L-Asp substrate (A) at pH 7.0 (a), pH 7.5(b), pH 8.0(c), and pH 8.5 (d); or with L-Ser substrate (B) at pH 7.0 (e), pH 7.5(f), pH 8.0 (g), pH 8.5 (h). L-Ser (13.3 mM) or L-Asp (26.7 mM) was incubated with 1 μ g DAR1 and cofactors in 150 μ l reaction in 50 mM Tris-HCl with various pH at 30 $^{\circ}$ C for 2.5 h. Reprinted with permission from reference 41.

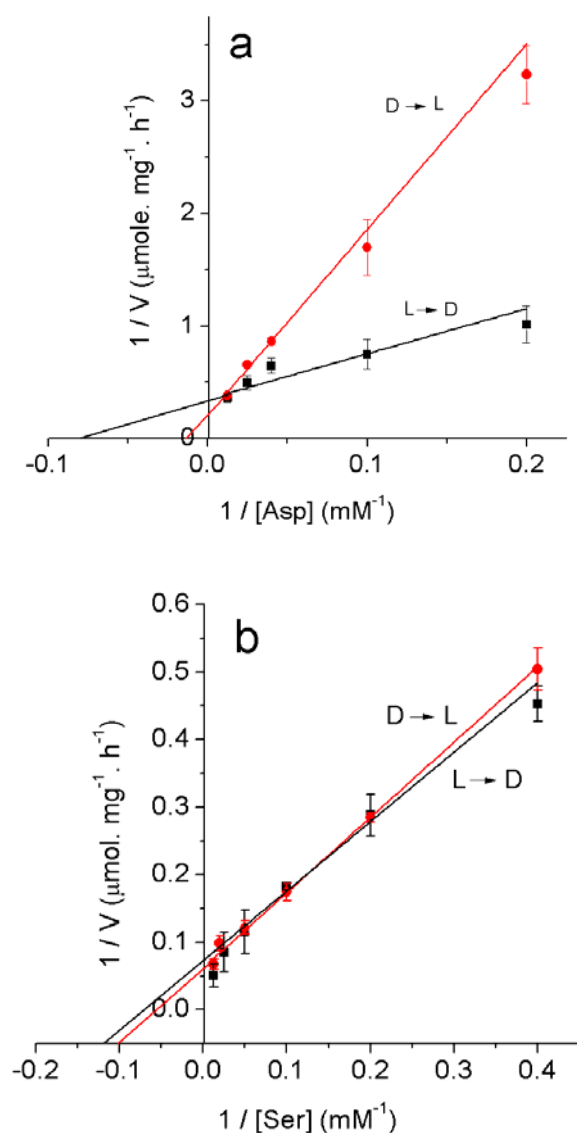


Figure 3.7 Lineweaver-Burk plots of DAR1 racemase kinetics. Racemase assay with various concentrations of Asp or Ser substrates were performed and conversion products were quantified as described in the Materials and Methods. Inverse reaction velocities were plotted against corresponding inverse substrate concentrations and fitted as linear curves with Excel to produce the Lineweaver-Burk plots for Asp (a) and Ser (b) substrates. Reprinted with permission from reference 41.

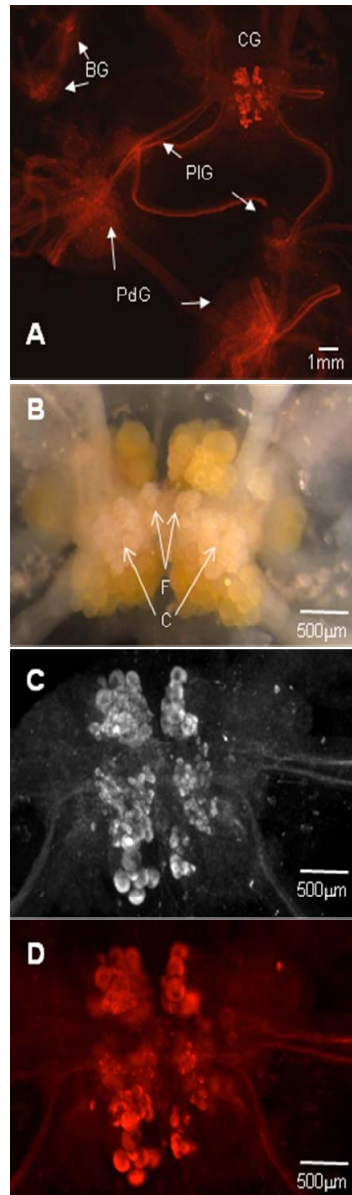


Figure 3.8 DAR1-like immunoreactivity localization in the *Aplysia* CNS. Whole-mount staining of the CNS (except abdominal ganglia) with rabbit anti DAR1 serum shown as a rhodamine fluorescence micrograph (a); BG: (buccal ganglia); CG (cerebral ganglia); PdG: pedal ganglia; PlG: pleural ganglia. Light micrograph of desheathed CG control without staining (b); enlarged CG image from (a) showing rhodamine fluorescence micrograph (c) and bright-field fluorescence micrograph (d). Reprinted with permission from reference 41.

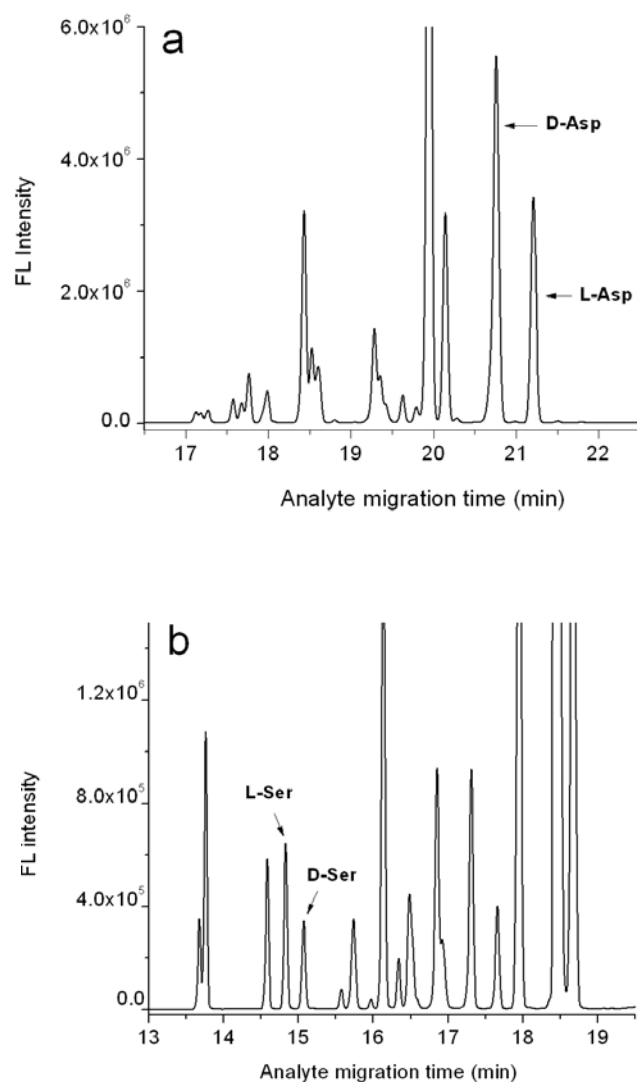


Figure 3.9 CE-LIF electropherograms of D-Asp and D-Ser in CNS neuronal F/C clusters. *Aplysia* cerebral ganglion F/C cluster neurons were dissected and subjected to amino acid separation with CE-LIF using optimal buffer for Asp separation (a) and for Ser separation (b). Reprinted with permission from reference 41.

3.7 References

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CHAPTER IV

DESCRIPTION OF APLYSIA RACEMASE β -ELIMINASE ACTIVITY BY COMPARATIVE STUDY OF APLYSIA RACEMASE AND SERINE DEHYDRATASE

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4. 1. Introduction

D-Ser is an endogenous co-agonist of the NMDA glutamate receptor, modulating receptor activity by binding to the glycine-binding site in NR1 subunit of NMDAR (1). The neurotransmitter/neuromodulator can affect synaptic plasticity of NMDAR (2). Aberrant D-Ser metabolism has been linked to some neurodegenerative disorders for example,

amyotrophic lateral sclerosis (3) and schizophrenia (4). D-Ser is synthesized from L-Ser by SR in mammalian neurons and glia (1). Knowledge of serine racemase structure and function is important to understand D-Ser biosynthesis and regulation, and more importantly D-Ser signaling pathways *in vivo*.

Eukaryotic SR (EC 5.1.1.18) and aspartate racemase (DR, EC 5.1.1.13) belong to pyridoxal-5'-phosphate (PLP)-dependent enzyme fold-type II (5) and they are structurally similar to *E. coli* threonine dehydratase (EC 4.3.1.19) (6). To date, only four animal SR have been characterized including rat, mouse and human SR (1, 7, 8) and DAR1 from *Aplysia californica* (9). Serine racemase converts between Ser enantiomers in an ATP-Mg dependent manner and tend to favor L-Ser over D-Ser substrate.

Besides racemase activity, eukaryotic serine and aspartate racemases display L-Ser dehydratase activity (SDH, EC 4.3.1.17) via β -elimination mechanisms (10-12). SDH degrades L-Ser to pyruvate and ammonia by a two-step reaction. The first step is enzymatic and PLP-dependent, leading to the elimination of β -hydroxyl group from serine to form aminoacrylate intermediate which undergoes rapid nonenzymatic hydrolysis to produce pyruvate and ammonia. The terms β -eliminase and dehydratase have been used interchangeably in past literature. L-Ser racemization and dehydratization occurs at the same

catalytic site of the mouse serine racemase (13) The catalysis steps for both reactions have been clearly depicted by Foltyn et al. (14). An L-Ser substrate bound to a SR can either become D-Ser by racemization or lead to pyruvate and ammonia by dehydration. While the racemization reaction is reversible, β -elimination is unidirectional.

The apparent competition between two activities for the same L-Ser substrate by a SR is puzzling. Furthermore, that the faster dehydratase activity and slower racemase activity usually observed with eukaryotic SR has raised questions about which activity plays a physiological role or under which conditions the racemization activity is physiologically important.

As the enzyme products D-Ser (or D-Asp) have well characterized biological activities (15-18), the physiological function of β -elimination of these racemases is under debate. There are at least three hypotheses to explain the β -elimination activity of the serine racemases. One hypothesis suggests that β -eliminase activity serves as a link between D-Ser transmitter production and neuron energy metabolism(19). This explains the dilemma of co-production of D-Ser and pyruvate from L-Ser from a SR; this hypothesis fits when considering that ATP is required for serine racemase activation (9, 10), and D-Ser transmission, and that subsequent cell activation consumes more energy than the resting state. However, it is not clear how

much and how quickly the β -eliminase activity of a racemase can contribute to the change of a cellular energy level. An unresolved question is why some racemase also dehydratizes L-Thr that forms 2-oxobutanoate, a compound does not contribute to energy metabolism (<http://www.genome.jp/kegg/pathway.html>). A second hypothesis proposes that serine racemase can serve as a metabolic enzyme to degrade D-Ser in mammalian forebrain where D-amino acid oxidase is absent (14). The most recent hypothesis agrees that β -elimination limits D-Ser levels by degrading D-Ser and its precursor L-Ser but also provides a “serine shuttle” model to explain how a neuron can solve the dilemma of D-Ser being made and destroyed at the same time (20). But D-Ser degradation models do not explain why bivalve aspartate racemase also has an L-Ser dehydratase activity (12), and perhaps more importantly, why a serine racemase limits an already slow serine racemase activity by degrading its product D-Ser and precursor L-Ser, especially as other enzymes exist that have dehydratase activity alone.

We have recently described the first invertebrate serine racemase DAR1 from *Aplysia californica* CNS (9), with this enzyme also having significant Asp racemase activity. As shown below, the enzyme shares many protein and enzyme features with mammalian serine racemases and has a specific L-Ser dehydratase activity. Because the localization and activity of specific *Aplysia* neurons can be studied, enzyme function can be linked to higher order network functions. DAR1 appears an enzyme well suited to studying the β -elimination activity of eukaryotic racemase to address issues of its physiological functions. During the

search for serine/aspartate racemase in *Aplysia*, we also located a putative serine dehydratase gene *acsdh* in an *Aplysia* CNS EST library. The gene is expressed in the *Aplysia* cerebral ganglion where DAR1 is located. Enzyme assay with recombinant AcSDH protein confirmed that it is an L-Ser dehydratase with weaker L-Threonine (L-Thr) dehydratase activity but no racemase activity. AcSDH has hence provided us with an enzyme to compare the function and localization of the L-Ser dehydratase activity of DAR1. We compared the two *Aplysia* protein sequences, their L-Ser dehydratase activities under different conditions and enzyme kinetics. Our results show that AcSDH is a robust L-Ser dehydratase whereas DAR1 L-Ser dehydratase activity is reduced compared to AcSDH, suggesting that the racemase activity is more significant than the creation

4.2. Materials and Methods

4.2.1 ORF Cloning of *acsdh* cDNA

Restriction enzymes were purchased from NEB (Ipswich, MA) and other cloning reagents were purchased from Invitrogen (Carlsbad, CA); enzyme reaction cleaning kits and miniprep reagents were purchased from QIAGEN. The *dar1* cDNA cloning from *Aplysia* CNS RNA was described by Wang, et al. (9). The cDNA of *acsdh* was initially cloned from *Aplysia* CNS EST library of juvenile *Aplysia* by Dr. Moroz at University of Florida and given us as an ORF fragment in pCR®2.0-TOPO® vector in a bacterial stock. But the ORF of *acsdh* cDNA was re-cloned by RT-PCR for expression vector cloning using forward PCR primer: 5'-GGCTCGAGATGGATTCCAAACCAGAT-3' (the XhoI site underlined) and reverse primer

5'-CCTGATCATATGCCAAAGTCTGCCTT-3' (the BclI site underlined) and *pfuTurbo* DNA polymerase (Agilent Technologies) with 55 °C annealing temperature. The PCR product was cloned into PCR®4-TOPO® vector and transformed into TOP10 cells and sequenced. Final clone used for expression vector construction was named 418M.

4.2.2 Protein Expression and Purification

Bacterial expression vector pET-15b, BL21(DE3) competent cells, NiNTA resin, protease cocktail, BugMaster Mix bacterial lysis buffer were purchased from EMD4Biosciences (Gibbstown, NJ). HiPrep Sepharacryl 200 HR 16/60 sizing column and low molecular weight standard kit for gel filtration were bought from GE Healthcare Life Sciences (Piscataway, NJ). The *acsdh* expression vector was constructed by digesting 418M plasmid with XhoI and BclI restriction enzymes and cloned the XhoI and BclI fragment between XhoI and BamHI sites of pET-15b. The resulting vector was transformed into TOPO 10 cells, sequenced and then transformed into *E. coli* BL21 (DE3) protein expression cells. AcSDH expression and purification were carried out by following the same procedures as those for DAR1 as reported by us before (9).

4.2.3 Protein Homology Analysis

The protein sequences of 13 PLP-dependent enzymes were downloaded from <http://www.ncbi.nlm.nih.gov> website. Bioinformatics analysis was conducted at SDSC Biology Workbench website: <http://workbench.sdsc.edu/>. DAR1 and AcSDH sequence were

aligned with ALING program; multiple protein sequence alignments were performed with ClustalW program and outputted as rooted phylogenetic dendrogram or inferring evolutionary tree by Phylip's Drawgram.

4.2.4 Racemase Assay

All chemicals are highest grades and purchased from SIGMA-Aldrich (St. Louis, MO) unless specified otherwise. DAR1 assay: 1 µg DAR1 (1 µg/µl) was added to 149 µl substrate mix containing 10 mM L-Ser in buffer mix containing 50 mM Tris-HCl with pH 7.0-10.0, 15 µl PLP, 2 mM DTT and MgCl₂ and 4 mM ATP at 30 °C for 2.5 h. The reactions were stopped by adding 5 % Trichloroacetic acid (TCA), and then 5 mM glycine was added to each sample for internal control of subsequent amino acid analysis by capillary electrophoresis laser induced fluorescence detection (CE-LIF). After 15 min on ice, the samples were centrifuged at 10,000 x g to remove protein precipitates, and the supernatants were extracted with an equal volume of water-saturated ether 2 times to remove TCA. The samples were then diluted 1:10 with 50 mM borate, pH 9.4, and 2 µl of the diluted samples were derivatized with 4 µl of 8 mM naphthalene-2,3-dicarboxaldehyde (NDA, Invitrogen) and 4 µl of 40 mM potassium cyanide (KCN) at RT for 60 min. The reaction was stopped by diluting the samples with 50 mM borate pH 8.5, and the samples were immediately frozen on dry ice and subjected to CE-LIF analysis within 24 h as described by Wang et al. (9). AcSDH assay: 1 µg of AcSDH (1 µg/µl) was added to a substrate mix containing one of the four amino acids (L-Ser, L-Asp, L-Ala, L-Glu) at 20 mM and 100 mM concentrations in a total 100 µl of reaction containing 50

mM Tris, pH 8.5, 20 μ M PLP and 2 mM DTT at 30 °C for 2.5 h. Negative controls contained no enzyme. Post-incubation treatment and NDA derivatization procedures were the same as for DAR1 racemase assay described above. The CE-LIF analysis of racemase products of D-Ser, D-Asp, D-Ala or D-Glu was described by Wang et al. (9).

4.2.5 β -eliminase Assay

All chemicals for assay were of highest purity and purchased from Sigma-Aldrich (St. Louis, MO), unless specified otherwise. L-(-)-threo-3-hydroxyaspartic acid (LT3HA) was purchased from TOCRIS Biosciences (Bristol, UK); L-Ser-O-sulfate (LSOS) and amino-oxyacetate (AOAA) were purchased from SIGMA-Aldrich. Serine or threonine dehydratase converts serine or threonine or their derivatives to keto acids and ammonia by β -elimination (13). The dehydratase activity can be measured by detecting α -keto acid products with 2, 4-dinitrophenylhydrazine (DNPH) in a colorimetric assay as described by Abe et al. (12) which was used with some modifications. An enzyme reaction started by adding 1 μ l (0.25-1 μ g) of an enzyme to a substrate mixture of 99 μ l, containing a substrate, cofactor PLP (15 μ M) and DTT (2-5 mM) for AcSDH assay, and in addition, $MgCl_2$ (2 mM) and ATP (1 mM) for DAR1 assay in 50 mM Tris-HCl, pH 8.5 buffer (unless specified otherwise). Except for temperature assays, all other assays were carried out at 30 °C. Enzyme reactions were incubated for 60 min and then stopped by adding 25 μ l of 25 % TCA. Negative controls contained no substrates. Every assay was repeated with three batches of enzyme preparations and technically triplicated. After TCA termination, color detection of keto acid was performed

by incubating samples with 70 μ l of 0.1% DNPH in 2 N HCl at 30 °C for 15 min, followed by the addition of 500 μ l of 3.75 M NaOH and incubation at 37 °C for 15 min. Finally, the samples were centrifuged at 8,800 x g for 10 min at RT, and 200 μ l of supernatants were measured at O.D. 445 nm for the absorption of 2, 4-dinitrophenyldrazone in 96-well flat bottom microtiter plates (Thermo Fisher Scientific) with Spectra_{max} Plus 384 spectrophotometer by Molecular Devices (Sunnyvale, CA). The O.D. 445 nm values were adjusted with negative controls and presented as Y-axis of values in graphs. The mean of three replicates and standard deviations are displayed.

4.2.6 Kinetics Assay of β -elimination Reaction

Optimal assay conditions tested were used to measure kinetics. For DAR1 assay, 1 μ l of DAR1 (500 ng/ μ l) was added to a 99 μ l reaction mix containing L-ser (2.5-80 mM), 15 μ M PLP, 2 mM DTT and MgCl₂, and 4 mM ATP in 50 mM Tris-HCl, pH 8.5 buffer and incubated at 30 °C (except for temperature assay) for 60 min. Reactions without substrate were blank controls. For AcSDH assay, 0.3 μ g of AcSDH (for L-Ser reactions) or 0.5 μ g of AcSDH (for L-Thr reactions) was added to a reaction mix containing either L-Ser or L-Thr, 15 μ M PLP and 5 mM DTT in 25 mM Tris-HCl, pH 9.0. The amount of enzyme was adjusted so that the pyruvate produced from the highest substrate concentration reactions were within the linear range of 0.05- 1.6 mM. Pyruvate standard series (0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mM) were co-incubated separately with enzyme samples in the enzyme assay buffer (without substrate) to produce a standard linear curve (O.D. 445 nm values versus pyruvate

concentrations). The linear equation of the standard curve was used to measure the pyruvate quantity produced by an enzyme reaction. Enzyme specific activity was defined as μ moles of pyruvate produced by 1 mg of enzyme during one hour incubation at 30 °C. Specific activities were plotted against substrate concentrations and the resulting curves were fitted with non-linear regression Michaelis-Menten model with OriginPro8 software (OriginLab, Northampton, MA) to produce K_m and V_{max} . The K_m and V_{max} from three enzyme batch repeats were averaged and presented with standard deviation in kinetics table.

4.3 Results

4.3.1 Protein Expression and Purification

SDS-PAGE analysis results of protein expression and purification are shown in Fig. 4.1. DAR1 and AcSDH recombinant proteins were both over-expressed in *E. coli* with IPTG induction. The proteins were purified from bacterial cells to homogeneity through nickel NTA affinity and gel filtration chromatography as described by Wang et al. (9). The recombinant proteins showed electrophoresis mobility of about 37.0 kDa as predicted. Molecular size calibration with gel filtration column indicated that both proteins formed dimers in solutions (data not shown). Purified protein preparations appeared yellow-green, suggesting the association of protein molecules with bacterial endogenous PLP cofactor.

4.3.2 Protein Homology Analysis

Figure 4.2 shows DAR1 (325 aa) and AcSDH (332 aa) predicted protein sequences and their optimal global alignment. The two proteins share 27 % homology including a conserved PLP-attachment motif surrounding putative PLP-interacting Lys56 (DAR1) or Lys50 (AcSDH) (12). Fifteen PLP-dependent enzyme sequences were aligned and alignment output was displayed a rooted phylogenetic tree shown in Fig. 4.3. The tree indicates that DAR1 is closest to bivalve DR and five eukaryotic serine and aspartate racemases form a family. AcSDH belongs to serine dehydratase family (SDH). Mouse aspartate racemase belongs to amino transaminase family. The three families are related to each other and to *E. coli* threonine dehydratase, biosynthetic and catabolic.

4.3.3 β -elimination Substrates and Competitors

Eleven amino acids were examined as substrates of DAR1 and AcSDH β -elimination activity. As predicted, only amino acids with hydroxyl functional groups were the substrates of β -elimination as shown in Fig. 4.4a. Both enzymes showed activity towards L-Ser but AcSDH activity is much stronger than DAR1; AcSDH also has a weaker activity towards L-Thr. As shown in Fig 4.4b, the two enzymes also have activity on L-serine-O-sulfate (LSOS), an L-Ser derivative; DAR1 also is very active towards LT3HA, an L-Thr/L-Asp derivative. DAR1 activity was 3 times and 50 times more active on LSOS and LT3HA than L-Ser, respectively. AcSDH showed a comparable activity on LSOS and L-Ser.

4.3.4. Enzyme β -elimination Activity Characterization

DAR1 and AcSDH L-Ser dehydratase activities were examined for PLP-dependency, thermo-stability, and the influence by reaction buffer pH and ion strength. Both enzymes were inhibited by AOAA, a competitor of PLP as shown in Fig 4.5a. The enzymes have a similar thermo-stability profile as shown in Fig. 4.5b. Activity increased as temperature went from 14 °C to 37 °C, but AcSDH lost activity at 45 °C and DAR1 stayed more active at 45 °C and only lost some activity at 50 °C, meaning AcSDH is more sensitive to higher temperature than DAR1. DAR1 and AcSDH showed different response to pH and buffer strength change, as demonstrated in Fig. 4.5c and Fig 4.5d, respectively. DAR1 displayed a pH-response pattern independent of Tris-HCl concentrations: sharp increase from pH7.0 to 7.5, slightly decreasing activity at pH 8.0, increasing activity at pH 8.5 and leveled or decreasing activity at pH 9.0. The enzyme also showed more activity in higher Tris-HCl concentration with an order of 100 mM > 50 mM > 25 mM. In contrast, AcSDH activity is clearly alkaline dependent and also favors lower Tris-HCl concentrations. Divalent cation (2 mM) and nucleotide (1 mM) effects had different effects on DAR1 and AcSDH. Fig 4.6a shows that MgCl₂ and CaCl₂ promoted DAR1 activity, ZnCl₂ and CuCl₂ inhibited it, and MnCl₂ had no effect on it; Fig 4.6b demonstrated that MgCl₂ had no effect on AcSDH activity; CaCl₂ was slightly stimulating it and MnCl₂, ZnCl₂ and CuCl₂ inhibited it. Fig 4.6c showed that ATP, ADP and GTP strongly promoted DAR1 activity, and AMP and GDP also promote DAR1 activity but to a much less extent. In contrary, all adenosine and guanosine nucleotides had more or less inhibition effects on AcSDH with strongest inhibition effects observed with ATP, ADP and GTP as

demonstrated in Fig. 4.6d. At 4 mM nucleotide concentrations, the inhibition effects to AcSDH were stronger (data not shown).

4.3.5 Alkaline Dependence of D-Ser Production by DAR1

DAR1 L-Ser racemase activity showed increased size of D-Ser peaks as assay buffer 50 mM Tris-HCl went from pH7.0-10.0 as shown in CE-LIF electrophoregrams (Fig.4.7a) or as normalized D-Ser peak values in 25 mM, 50 mM and 100 mM Tris-HCl buffers with pH7.0-9.5 with 0.5 pH unit increment in the line graph as in Fig 4.7b. As shown in the line graph, D-Ser production continued to increase between pH9.0-9.5 for 50 mM and 100 mM Tris buffers while 25 mM buffer gave a peak value at pH9.0. Among three buffers tested, 50 mM Tris-HCl buffer showed a stable and consistent better performance than that in 25 mM and 100 mM Tris-HCl buffers.

4.3.6 Enzyme Kinetics Parameters

Fig. 8a demonstrated a linear relationship between the concentration series of standard sodium pyruvate (0-1.6 mM) and corresponding O.D. 445 nm values obtained from the color assay described in the Material and Methods. The standard curve was used to calculate dehydratase activity reaction products α -keto acids. The representative substrate saturation curves of DAR1 reaction with L-Ser substrate (Fig 4.8b), or AcSDH reaction with L-Ser (Fig 4.8c) or L-Thr (Fig. 4.8d) substrates were shown with overlaying Michaelis-Menten non-linear regression fittings. Table I shows the kinetics parameters of L-Ser racemase and L-Ser

dehydratase reaction catalyzed by DAR1, and L-Ser and L-Thr dehydratase reactions catalyzed by AcSDH under the experimental conditions used. L-Ser dehydratase activity is 3.5 times faster than L-Ser racemase activity when determined by enzyme turnover number k_{cat} . For AcSDH, L-Ser dehydratase activity is 7 times faster than L-Thr dehydratase activity. When L-Ser dehydratase activity was compared, AcSDH was 20 times faster than DAR1. Finally, the catalytic efficiency (k_{cat}/K_m) of AcSDH on L-Ser dehydratization is 7 times higher than DAR1.

4.4 Discussion

The two *Aplysia* enzymes, DAR1 and AcSDH are functionally related but how close are they to each other and to other PLP—dependent enzymes? Protein homology analysis by multiple sequence alignment of 15 PLP-dependent enzymes separated DAR1 and AcSDH to two different families as shown in the inferring evolutionary tree as shown in Fig. 4.3. DAR1 belonged to racemase family which members include bivalve DR (EC 5.1.1.13) and mammalian SR (EC 5.1.1.18) while AcSDH was grouped with eukaryotic serine dehydratase (EC 4.3.1.17, E) and threonine dehydratase (EC. 4.3.1.19), or simply named serine dehydratase (SDH) family. Mouse DR represents a distinct eukaryotic racemase which belongs to aspartate transaminase (EC 2.6.1.1) family and is not discussed further. The racemase and SDH families, and *E. coli* threonine dehydratase (THD, EC 4.3.1.19), both biosynthetic and catabolic enzymes (EC 4.3.1.19) seemed to be closely related but evolved divergently from a common distant ancestor. They share a structural similarity of fold type II within PLP-

dependent enzymes and the same catalytic function as β -eliminases. It is possible that the distant ancestor was a prokaryotic serine or threonine dehydratase which passed its β -eliminase activity to the racemase and SDH families. In bacteria, serine and threonine dehydratases are usually two separate enzymes but the eukaryotic SDH family members have both serine and threonine dehydratase activities. It is likely, therefore, the ancestor added one more β -elimination substrate to become today's dual-substrate dehydratase while racemase family acquired racemase function and evolved into the today's racemase enzyme. Although both families possess L-Ser dehydratase, they are probably to be selected by nature to serve different physiological functions: SDH family to make keto acids from L-Ser and L-Thr, and the racemase family to synthesize D-Ser or D-Asp.

DAR1 and AcSDH demonstrated different substrate profiles. Both enzymes can dehydrate L-Ser but DAR1 is inactive to L-Thr while AcSDH has a moderate L-Thr activity. DAR1 is thus different from mouse SR which substrates include L-Ser, D-Ser and L-Thr (14). More importantly, DAR1 has Ser and Asp racemase activities (9) but AcSDH had no detectable racemase activity towards L-Ser, L-Asp, L-Ala and L-Glu substrates under the same assay conditions as its L-Ser dehydratase assay confirming that this is a SDH and not a racemase. We also examined two chemical compounds LSOS, an L-Ser derivative, and LT3HA, an L-Asp derivative with a hydroxyl group as enzyme dehydratase substrates. The compounds are strong substrates of mouse racemase β -elimination activity (9, 12, 14) and they could non-

competitively inhibit mouse SR racemase activity (13). DAR1 showed stronger activity towards both compounds than L-Ser, a result also observed with mouse SR with LSOS (21) or that with bivalve DR with LT3HA (12). Differently, AcSDH only had activity towards LSOS with a comparable level to L-Ser substrate. The different substrate profile suggests that DAR1 and AcSDH have different catalytic binding pockets. LT3HA occurs in some polypeptide antibiotics which are blockers of excitatory amino acid transporters (22). But we haven't found any literature showing both compounds are endogenous in animals.

Determining the conditions of activity are important indicators to enzyme functions. Both DAR1 and AcSDH β -elimination activities were inhibited by amino-oxyacetic acid (AOAA). DAR1 and AcSDH L-Ser dehydratase activity favor different reaction conditions. In 25 mM, 50 mM and 100 mM Tris-HCl buffers, DAR1 demonstrated pH-dependent activity with a sharp increase from pH 7.0 to pH 7.5 and a complex activity profile between pH 7.9-9.0 (Fig. 4.5c). In addition, for any given pH DAR1 was more active in high ionic strength than lower ionic strength buffers. Of course, *Aplysia* cells contain a high ionic strength (>500 mM cationic concentration in their cytoplasm). Quite distinct, AcSDH is clearly alkaline dependent showing continued increasing activity from pH 7.0-9.0 for the tested three ionic strength buffers (Fig. 4.5d); and the enzyme was more active in lower ionic buffer, opposite to DAR1. The different ionic preferences suggest that the two enzymes might achieve their

optimal activities in separate subcellular compartments in a cell or under different physiological conditions. More cellular/molecular physiology work is needed to confirm this.

Eukaryotic racemase activity dependence on divalent cations and nucleotides is well documented (9, 10, 12). In this study, we examined these effectors on L-Ser dehydratase activity of DAR1 and AcSDH. We have examined five divalent cations and eight nucleotides that are frequent enzyme stimulation or inhibition effectors. The two enzymes showed distinct reactions to Mg^{2+} , Ca^{2+} and Mn^{2+} and have the opposite reactions to ATP, ADP and GTP. The different reactions to the effectors suggest that the enzymes are activated by different metabolic conditions thus they likely have different physiological functions. For example, AcSDH plays an important role in pyruvate metabolism which can lead to energy production thus the enzyme can be feedback inhibited by energy molecules ATP, ADP and GTP; DAR1 is probably involved in a cellular process that requires a sufficient cellular energy level to be activated, for example, neuronal excitation or signal transduction activation.

We measured K_m and V_{\max} of DAR1 and AcSDH and calculated parameter k_{cat} and k_{cat}/K_m for both enzymes. Based on the k_{cat} , DAR1 was 3.5 fold faster in generating pyruvate from L-Ser than producing D-Ser from L-Ser under the experimental conditions used. The faster L-Ser β -elimination reaction than L-Ser racemase reactions was also observed with mouse SR (10). The two enzymes showed a great difference in catalyzing L-Ser to pyruvate and ammonia per unit time: AcSDH was 20-fold faster than DAR1. In addition, AcSDH k_{cat}/K_m is also at least 7

times larger than DAR1. Both kinetic parameters strongly indicate that AcSDH functions as β -eliminase with a robust L-Ser dehydratase activity while DAR1 L-Ser dehydratase activity is weaker, lending credence to the importance of its other cellular function.

The DAR1 dehydratase activity pH profile (Fig. 4.5c) showed that its pyruvate production rate had a complex pH dependence profile as the buffer pH increased from neutral to basic and DAR1 racemase pH-dependent profile showed a gradual upward trend as pH went from 7.0 -10.0 with 0.5 pH increments as shown in CE-electrophoregrams Fig. 4.7a or the line graphs in Fig. 4.7b. In parallel, mouse SR L-Ser dehydratase reached peak activity at pH 8.0 but its L-Ser racemase activity did not reach peak value until pH 9.0 (21). The different responses to alkaline pH by the two activities of a serine racemase are interesting. Mammalian cytosolic pH is 7.0-7.4 (23) but at this pH range, mouse SR racemase activity was only 30% of its peak activity at pH 9.0 (21). Molluscan intracellular pH is similar 7.2 (24). But at pH 7.0 with 50 mM Tris-HCl buffer, D-Ser production by DAR1 was only 21% of that at pH 10.0 (with CE internal control normalized value shown in Fig 4.7b). Therefore, to achieve faster conversion, the racemase should be located in an cellular compartment with a basic milieu, or should such a basic environment be absent, a racemase might raise the local pH of the surrounding enzyme catalytic pocket by creating ammonia from Ser/Thr breakdown by its β -elimination activity. Therefore, one function of the β -elimination is not directly involve energy metabolism but produces ammonia from Ser and/or Thr substrates. This hypothesis can explain why two apparent competing reactions coexist in a racemase; the β -elimination

activity is probably not to compete with racemase activity for L-Ser substrate to make pyruvate or breakdown D-Ser to prevent neurotoxicity, rather to assist racemase activity to achieve its maximum activity by creating an alkaline environment by producing ammonia.

In addition, both bivalve and DAR1 aspartate racemase activities are also promoted under alkaline conditions and both enzymes have L-Ser dehydratase activities (9, 12). Second, mouse SR β -elimination substrates include L-Ser, D-Ser and L-Thr. Ser and Thr breakdown involve the formation of pyruvate and 2-oxobutanoate, respectively, plus ammonia. Pyruvate can be an energy source via the citric acid cycle but 2-oxobutanat enters a non-energy related metabolic pathway (<http://www.genome.jp/kegg/pathway.html>). Given these reasons, we expect that energy and/or controlling D-Ser levels may not be primary function of β -elimination activity of a serine racemase. Does the generation of ammonia used to enhance racemase activity? Further studies are needed with other eukaryotic serine racemases to support this hypothesis.

4.5 Conclusions

Like other eukaryotic serine or aspartate racemases, DAR1 also has L-Ser racemase and L-Ser dehydratase activities. The former activity produces D-Ser from L-Ser and the later degrades L-Ser to pyruvate and ammonia. While D-Ser physiological functions in animals are well documented, it is not clear what the physiological function of the L-Ser dehydratase activity is. To address the issue, a comparative study with DAR1 and Aplysia L-Ser dehydratase AcSDH

were conducted. Both enzymes were in *Aplysia* CNS and both have L-Ser dehydratase activities. Protein homology analysis, enzyme characterizations under different conditions and enzyme kinetics studies have revealed that they belong to eukaryotic racemase family and L-Ser dehydratase family, separately. Their L-Ser dehydratase activities showed distinct behaviors at alkaline pH conditions, favored low or high buffer strengths and responded to Ca^{2+} , Mg^{2+} or Mn^{2+} effectors differently. They also reacted to nucleotide ATP, ADP and GTP in an opposite manner. The different characters displayed by the two enzymes have strongly suggested their different physiological functions. Enzyme kinetic parameter analysis demonstrated that AcSDH has 20 folds larger k_{cat} and 7 folds larger $k_{\text{cat}}/k_{\text{m}}$ than DAR1. Together, the data strongly suggest that AcSDH plays a role in keto acid metabolism and/or energy metabolisms by producing pyruvate from L-Ser by its robust L-Ser dehydratase activity and 2-oxobutanoate from L-Thr by its moderate L-Thr dehydratase activity. DAR1 showed a much weaker L-Ser dehydratase activity compared to that of AcSDH, thus it might serve different cellular functions other than pyruvate metabolism. Since DAR1 L-Ser racemase is alkaline dependent, its L-Ser dehydratase activity can create an alkaline environment for its racemase activity by producing ammonia. Therefore, the physiological function of DAR1 β -eliminase activity is probably not to compete with its racemase activity rather to assist it. The pH hypothesis needs more studies with other serine and aspartate racemases to prove.

4.6 Figures

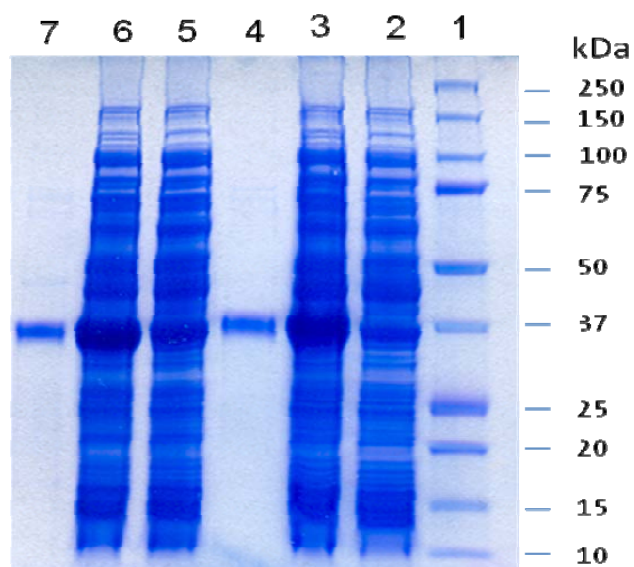


Figure 4.1 Protein expression and purification. Bacterial lysates prepared from protein expression host cells and purified proteins were subjected to SDS-PAGE with 4-20% Precise™ protein gel under reducing conditions. The Coomassie blue staining of the gel: 5 µl Precision Plus Protein™ Standards (lane 1); 5 µl lysates from non-induced and IPTG induced AcSDH culture (lane 2 and 3) or DAR1 (lane 5 and 6) samples; 1 µg of purified recombinant AcSDH (lane 4) or DAR1 (lane 7).

```

AcSDH      -----HDSKPDYCATDNKEKLYNETPVHYTSFPLSEHAGFKVYLKLDNLQPSGSEFKIRGI
DAR1       HAASCQVTFVDVLKALERISPIIHKTPIFTSEQANRKCGRQLFFKCEMFQKSGSEFKARGA
           :      * : . . : *::: * . . . * :::: *::: *::: *::: *:::
AcSDH      SMNLOKQIDRG-DSEHYTCASGGWAGHAAAHASKQLGIPCTIIVFPQTTFEFYMERLEWLG
DAR1       LNAVLYKQQCVKPMVNGVVTSSGNHGCALAWAAQRANLPCCVWFQHAAPDYKKAALRGYG
           * : * : : : * : * * * * * : : : *::: *::: *::: *::: *:::
AcSDH      AEVYVNGSVYDEAKKLAVELGSPCHLIPAFENPDIVECHASLINESSPQNSERPDLVI
DAR1       AELLECGPKPSDRNEACDKVQDDRMFELIPPYCHVDVIAGQGTIAVELLEQVP-FLDAIL
           ** : * . . : : . : . . . . . . . . . . . . . . . . . . . . . .
AcSDH      TCVGGGCLLAGIVQCHRDVGVDEVVPLANETHGANCFTAAATAAGEAVFIP---AITSIAK
DAR1       VPISGGCHSSGICIAAKTIKP-DIKIFIVAPKCK-BLECLRTGHRPWEGPPQYLDTIAD
           . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
AcSDH      SLGSLIVSPQTLEYTKQARPRILNENVSINQVIDACVRFSDOHRFLVEPACGACLLAAVYS
DAR1       GIRLQQTGYITTPILHELAEKDVFEHS-DEEIIEGHKFSFERHKLVIETAAGAEVAAAFS
           . : . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
AcSDH      NVVKQLQDSGKLGTKSVLVVVCQGSIVSTAVNEKEKADFGI
DAR1       DELEKNDP-----DLKNVGVILCGNLDIEHLPF-----
           : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :

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Figure 4.2 DAR1 and AcSDH protein sequence alignment. Two protein sequence alignment was carried as described in the Material and Methods. Asterisk: identical amino acids; colon: conserved amino acids; period: similar amino acids; underlined: putative PLP-binding motif; red colored K: putative PLP binding lysine. Sequence homology: 27 %.

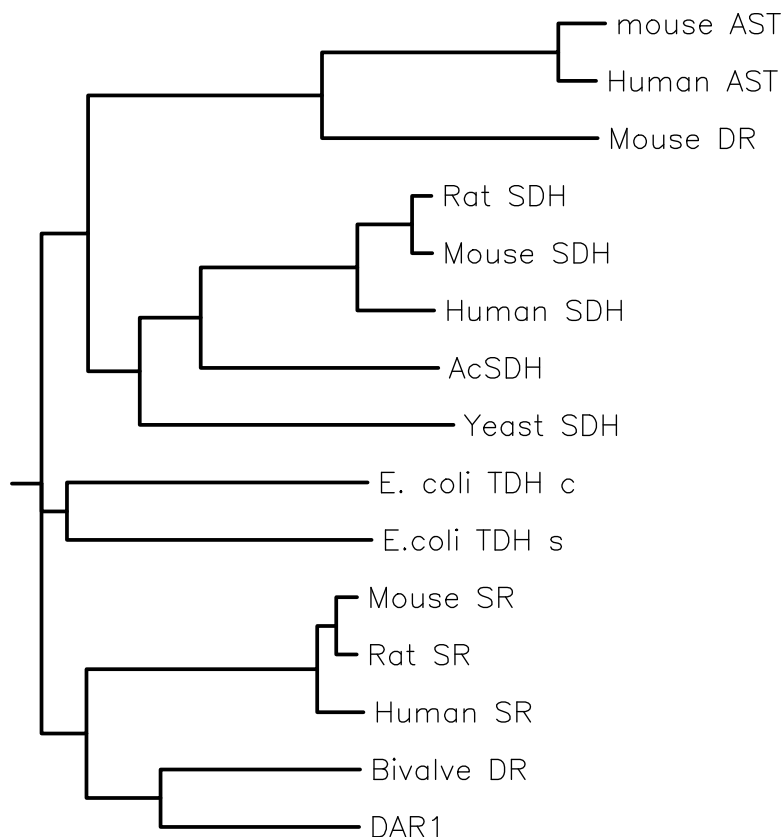


Figure 4.3 Inferring phylogenetic tree of 15 PLP-dependent enzymes. The rooted phylogenetic tree was generated as described in the Materials and Methods. TDH: threonine dehydratase; SDH: serine dehydratase; AST: aspartate aminotransferase; SR: serine racemase; DR: aspartate racemase; mouse SDH(NP_663540); rat SDH(NP_446414); human SDH(NP_006834); AcSDH(*A. californica* serine dehydratase: gb_AAU05774.1); yeast SDH (gb_AAA35040.1); human AST(NP_002070); mouse DR (25); *E. coli* biosynthetic TDH (NP_418220); *E. coli* catabolic TDH (AP_003665); DAR1(*A. californica* D-amino acid racemase 1, AC_HM776055); bivalve DR(BAE78960.1); rat SR(NP_942052); mouse SR(NP_001156783); human SR(NP_068766).

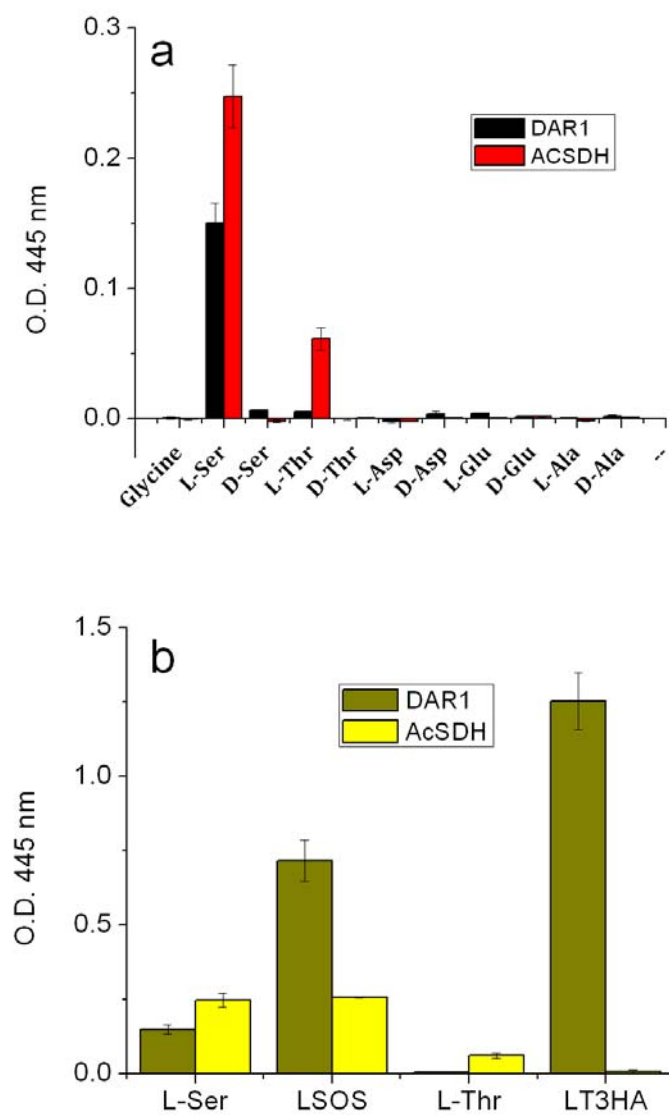


Figure 4.4 Dehydratase activity substrates and competitors. Eleven amino acids (10 mM) and L-Ser derivative, LSOS (10 mM) and L-Asp derivative, LT3HA (1 mM) were co-assayed with 500 ng of DAR1 or AcSDH in dehydratase assay as described in the Materials and Methods. Amino acid substrates results are shown in (a) and comparative results of L-Ser/L-Thr versus LSOS and LT3HA were shown in (b).

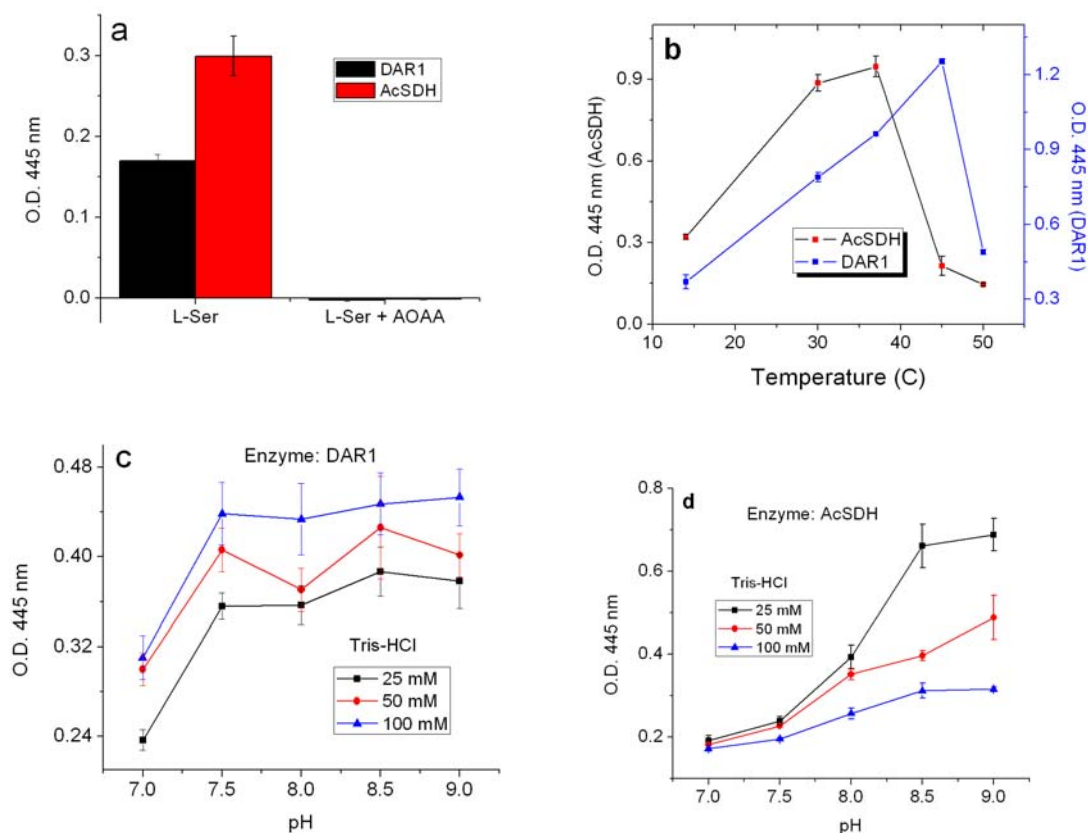


Figure 4.5 Enzyme characterizations. Inhibition of PLP-dependent SDH activity by AOAA (4a): L-Ser (10 mM) was incubated with 500 ng of enzyme in the presence of 1 mM AOAA in reaction buffers. Thermo-stability profiles of SD activity (4b): L-Ser (50 mM) was incubated with 250 ng AcSDH or 1 μ g DAR1 at 14 $^{\circ}$ C, 30 $^{\circ}$ C, 37 $^{\circ}$ C, 45 $^{\circ}$ C and 50 $^{\circ}$ C. Ion strength and pH effects on SD activity of DAR1 (4c) and AcSDH (4d): L-Ser (10 mM) was incubated with 1 μ g DAR1 or 0.5 μ g AcSDH in 25 mM, 50 mM, or 100 mM Tris-HCl buffers with pH 7.0, 7.5, 8.0, 8.5 or 9.0.

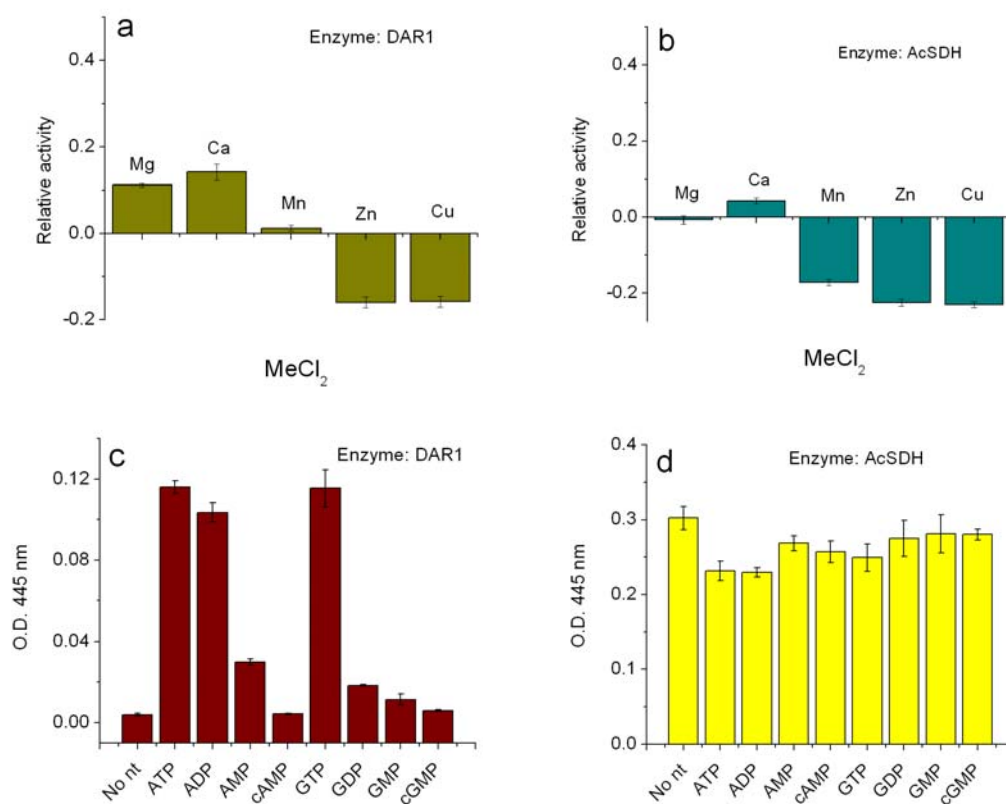


Figure 4.6 Cation and nucleotide effects on enzyme activity. Divalent cation assay: metal chloride salt (2mM) was incubated with 10 mM L-Ser and 1 μ g DAR1 (5a) or 0.5 μ g AcSDH (5b) in 50 mM Tris-HCl, pH 8.5 containing 15 μ M PLP and 2 mM DTT buffer. DAR1 assay also included 1 mM ATP. Y-axis: relative activity (O.D. 445 nm values were adjusted with no cation values). Nucleotide assay: adenosine and guanosine nucleotide was incubated with 40 mM L-Ser and 400 ng of DAR1 (5c) or AcSDH (5d) in the Tris-HCl buffer mix above. DAR1 reactions also included 2 mM MgCl_2 . The O.D. 445 nm values were adjusted with no enzyme controls.

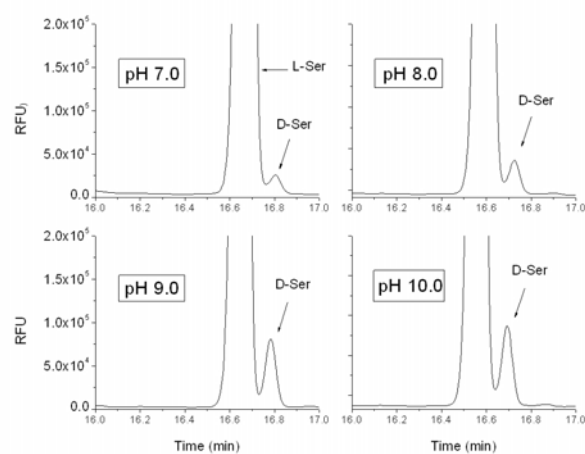
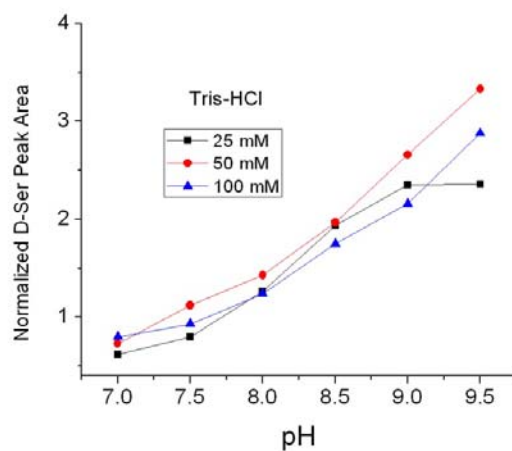
A**B**

Figure 4.7 Alkaline pH-dependence of DAR1 D-Ser production. Results shown as electrophoregrams (A) or shown as normalized D-Ser peak values (B). L-Ser racemase assay and CE-LIF detection of D-Ser products were described in the Materials and Methods in Chapter III, section 3.2.7. CE-LIF detection results are shown by CE-electrophoregrams for racemase reactions conducted in 50 mM Tris-HCl, pH 7.0 (a), pH 8.0(b), pH 9.0 (c), and pH 10.0 (d). Normalized D-Ser peak area: D-Ser peak area was divided by glycine peak area in the same CE-LIF electrophoregram.

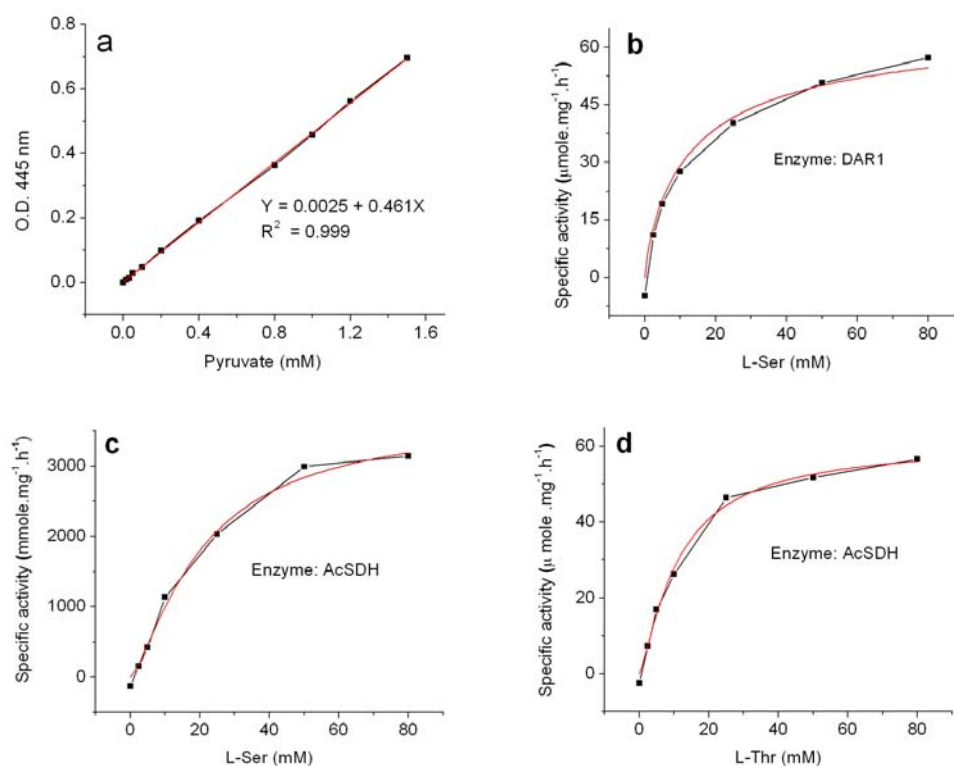


Figure 4.8 Enzyme β -elimination kinetics curves. Pyruvate standard curve and linear fitting (6a), representative enzyme kinetics curves with Michaelis-Menten fittings: L-Ser kinetics by DAR1 (b), L-Ser kinetics by AcSDH (c), and L-Thr kinetics by AcSDH (d).

4.7 Table

Table 4.1 DAR1 and AcSDH kinetics parameters

Enzyme	Reaction	K_M (mM)	V_{Max} ($\mu\text{mol mg}^{-1} \text{h}^{-1}$)	k_{cat} (min^{-1})	k_{cat}/K_M ($\text{min}^{-1} \text{mM}^{-1}$)
DAR1	L-Ser \rightarrow D-Ser	*14.9 \pm 1.84	*11.0 \pm 0.61	6.8 \pm 0.2	0.56 \pm 0.1
	L-Ser \rightarrow pyruvate	15.28 \pm 1.2	80.8 \pm 6.32	25.18 \pm 1.95	1.65 \pm 1.6
AcSDH	L-Ser \rightarrow pyruvate	13.16 \pm 1.9	326.4 \pm 32.4	619.6 \pm 61.5	47.08 \pm 32.4
	L-Thr \rightarrow 2-oxobutyrate	12.17 \pm 0.97	61.6 \pm 3.8	76.69 \pm 4.7	6.3 \pm 4.8

*Serine racemase reaction K_M and V_{Max} were measured and described by (9, 12). The kinetics parameters of β -elimination reactions were determined by measuring α -keto acids (pyruvate or 2-oxobutanoate) in the color assay as described In the Materials and Methods. Recombinant protein molecular weights of 37.3kDa and 37.6kDa were used to calculate k_{cat} for DAR1 and AcSDH, respectively. One catalytic site per enzyme molecule was assumed. Values are means \pm SEM, N=3 (data collected with 3 batches of enzyme preparations).

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CHAPTER V

MISCELLANEOUS RESEARCH PROJECTS AND METHODS

Notes and Acknowledgements

The microdialysis experiment described in this chapter was in collaboration with Martha Gillette Laboratory which provided mouse microdialysis fluid samples for our CE-LIF analysis. We thank Abigail Salyer Laboratory for generously providing Southern blot buffer recipes and DNA hybridization equipments. We thank Division of Animal Resources for animal care in support for antibody production project; Immunological Resource Center (IRC) for technical assistance in hybridoma tissue culture work; and Protein Science Facility (PSF) for peptide synthesis. Both IRC and PSF are units of Roy J. Carver Biotechnology Center at University of Illinois. Animal procedures were conducted in compliance with animal care and usage guidelines established by Federal, State of Illinois and University of Illinois authorities. The work was supported by Award NO. NS031609 from the NINDS, National Institutes of Health (NIH), and by Award No. CHE-04-00768 and CHE-05-26692 from the National Science Foundation (NSF).

5.1 Introduction

This chapter includes three methods and two experiments and that are not included in the previous chapters. The two of the methods are alternative approaches that were used to clone

DAR1 5' UTR and 3' UTR. The third method was developed for performing DAR1 enzyme assay in 96-well plate format. These methods were integrated parts of my research efforts on DAR1 project thus necessary to be included in this dissertation. More importantly, these have become successful methods so that they can be used as reliable and useful references for future students who work on cDNA cloning or D-amino acid study with enzyme approach. The last two projects are distinct. One of the two experiments included in the chapter is to develop a monoclonal antibody for detection/characterization of D-amino acid containing peptides (DAACPs) in animal tissues. The experiment is an integrated part of D-amino acid research efforts in the Sweedler group and thus worth being described in the dissertation. Another experiment was a part of a collaboration project on mammalian circadian system study with Martha Gillette Laboratory. Although the project was not associated with DAR1 project, it provided me a necessary opportunity to gain knowledge and hands-on experiences on CE-LIF system which was used heavily in DAR1 enzyme assay later. In addition, the data derived from the CE-LIF analysis provided useful information for designing following experiments on L-glutamate effects on mouse circadian system. The three methods related to DAR1 projects are described first and then followed by two experiments, DAACP antibody production and CE-LIF analysis of microdialysis fluid samples looking for neurotransmitter L-glutamate change in suprachiasmatic nucleus (SCN).

5. 2 Cloning of 5' UTR with Terminal Deoxynucleotidyl Transferase

The 5' UTR of dar1 cDNA was first cloned using RNA ligase-mediated 5' RACE method as described in the Chapter III, section 3.2.2. The resulting 5' UTR fragment only consists of 20 nucleotides and it is unusually short. Thus the 5' UTR needed to be verified by an alternative 5' RACE method. The method was used as the alternative method which was mentioned in Chapter III but is described in detail as follows.

To perform terminal deoxynucleotidyl transferase (TdT) mediated 5' RACE, A new batch of total RNA was prepared from CNS material from 4 fresh adult animals of 200 grams and RNA was extracted by TRIzol reagent as described in Chapter III, 3.2.2. RNA integrity was confirmed by Agilent Bioanalyzer before 5' RACE experiment. The alternative 5'RACE kit was purchased from Invitrogen and used by following product instructions.

The first strand cDNA was synthesized with 7.84 µg of total RNA, 2.5 pmoles of GSP primer (R952): 5'-GAT TCC CAC CAC ACA AAA TG-3' and 2 µl (200 units) of Superscript II reverse transcriptase in a 54 µl total reaction volume in 250 µl of PCR tubes for 50 min at 42 °C in a PCR machine. The newly synthesized cDNA was then immediately purified with S.N.A.P column and eluted into 50 µl of preheated 65 °C water. A dCTP tail was then added to the 3' end of the first strand cDNA by TdT enzyme. The tailed cDNA was then amplified with GSP R350: 5'-CGGATT GCA TTC TTC TTC AC-3' and commercial primer AAP: 5'-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3', and native Taq DNA polymerase with the following PCR reaction profile: 94 °C for 2 min for 1 cycle, 94 °C for 1

min, 55 °C for 30 sec, 72 °C for 2 min for 35 cycles and 72 °C for 10 min. The PCR product was re-amplified using nested PCR with GSP R202: 5'-GAC ACT TCA AAA CGG CAT TC-3' and commercial primer AUAP: 5'-GGC CAC GCG TCG ACT AGT AC-3' and native *Taq* with the following profile: 94 °C for 2 min for 1 cycle; 94 °C for 1 min, 55 °C for 30 sec, 72 °C for 1 min for 29 cycles; 72 °C for 10 min for 1 cycle. The PCR fragment was cloned into pCR4.0Topo vector and sequenced. The 5' UTR sequence cloned by the TDT method was the exact the same as 5' UTR obtained with RNA oligo-mediated 5'RACE method.

5.3 Southern Blot with Non-radioactive DNA Probe for 3' UTR Cloning

During complete cDNA cloning, 3' UTR cloning is the most challenging part. In fact, many novel gene cDNAs were published without 3' UTR. The reason is that 3' UTR cloning (by 3' RACE) is based on the fact that every mRNA transcript has a polyA tail. By using polyT primer, mRNA transcript can be reverse transcribed into cDNA which then can be amplified by PCR for subsequence cloning. However, because polyT primer can anneal to any nucleotide fragment that contains a stretch of adenine, specificity of 3'RACE is usually poor which was demonstrated by multiple PCR bands in a DNA gel. However, by using gene specific DNA probe, a target band can be easily identified from a Southern blot. Once the target band was identified, the band can be removed from a fresh DNA gel and cloning can be followed. During 3' UTR cloning of *dar1* cDNA, many non-specific band were seen, thus it was necessary to develop the method to aid 3' UTR cloning. Radioisotope labeled DNA probe was used in traditional Southern blot method but newer method uses non-radioactive DNA

probe. This section describes how to make non-radioactive probe by PCR method and how to use the probe to perform Southern blot experiment. The particular method described here was developed for making *dar1* probe based on some published methods.

The following procedure describes how to make PCR probe with DIG kit purchased from Roche Applied Sciences. The DIG Nonradioactive Nucleic Acid Labeling and Detection System (Roche Applied Sciences) were chosen to synthesize PCR probe. There are a few ways to incorporate DIG into a DNA probe. But PCR method was chosen because it can make highly sensitive DIG probes which can detect rare mRNA transcript. Specifically, 15bT90-31 plasmid (Reference chapter III) was used as PCR template which contains open reading frame of *dar1* gene. PCR primers 5'-CTTTCGTCGATGTGCTCAAA-3' (F-23, forward) and 5'-TCCACGGGCTTTAAATGAAC-3' (R-177, reverse) and Platinum Taq High Fidelity (Invitrogen) was used in PCR labeling reaction: 69 pg of 15bT90-31 plasmid, 0.2 μ M of each primer, 5 μ l of dNTP mix (normal NTP: DIG dNTP = 1:1), 5 μ l of 10 x Taq buffer, 1.5 μ l $MgCl_2$ (50 mM) and 0.75 μ l of Taq enzyme and dH_2O to 50 μ l were reacted under the following thermal profile: 95 °C for 2 min for 1 cycle; 95 °C for 30 sec, 52 °C for 40 sec, 72 °C for 30 sec for 39 cycles; 72 °C for 7 min. The probe (5 μ l) was examined on 1 % agarose gel to make sure that the probe incorporated DIG which can change PCR migration time. The DIG containing PCR band will move slower than normal PCR band of the same nucleotide size. Under the PCR reaction condition above, DIG probe was successfully made,

demonstrated by slightly higher molecular weight of DIG PCR band than non-DIG PCR band in agarose gel. The probe was frozen at -20 °C without any purification until hybridization.

Before Southern transfer, 3' RACE PCR reaction (10 µl) was resolved on 1 % agarose gel with DIG labeled molecular marker. The gel was stained with coomassie blue and a picture was taken. The gel was then treated with 0.2N HCl for 15 min with gentle shaking, rinsed twice with dH₂O, denatured with Denaturation Buffer (0.5 M NaOH/1 M NaCl) for 2 x 30 min, rinsed with dH₂O, soaked in 2 x 30 min in Neutralization Buffer, rinsed with dH₂O and then ready for Southern transfer.

The gel was transferred to positive charged nylon transfer membrane (GE Water & Process Technologies) by capillary method. To set up transfer assembly, soak the membrane in a crystal tray filled with 1 liter of Transfer Buffer (20 x SSC) with a gel cassette sitting in the buffer upside down and overlaid with a piece of 3 MM filter paper about the cassette width (i.d.) and with two sides overhanging in the buffer. On the top of the filter paper, DNA gel treated above was laid down and then the nylon member slightly smaller than gel (wetted in H₂O first) was laid down and then 3 pieces of 3 MM filter paper, then 6 pieces of thick filter paper, then 4 cm thick of paper towel, then a plastic plate was put on the top of the assembly, and finally a 250 ml glass bottle weighing 300-400 grams was put on the top of the plastic plate. The DNA band in the gel were allowed to slowly transferred to the nylon membrane through capillary force overnight for 16 h in 20 x SSC. After transfer was done, the nylon

membrane was removed from the assembly with forceps and put on a piece of Saran Wrap with DNA side (or the side that face the gel during transfer) up and then the DNA was cross-linked to the membrane by UV light for 2 x 15 sec with Strategene auto Cross-linker. The membrane now is ready for hybridization described below.

DNA hybridization was performed by following steps: membrane was put into a hybridization tube with 10 ml Prehybridization Buffer (6xSSC, Denhardt's solution, denatured Salmon Sperm DNA, formamide, EDTA, SDS) and incubated for 3-4 h at 42 °C oven with rotating balanced tubes. At the end of the prehybridization, add 20 µl denatured PCR DIG probe and allowed hybridization between DNA and the probe for overnight.

Post-hybridization wash and band detection were described below. The membrane was removed from hybridization tube and washed with 2 x 100 ml x 20 min with the following buffer: 2X SSC/0.1 % SDS and then 0.2X SSC/0.1 % SDS. The membrane was then washed with 100 ml of Washing Buffer (Maleic Acid, NaCl, pH 7.5) twice at RT for 2 min with gentle shaking and then incubated with 100 ml 1 x blocking buffer (2.5 % BSA or 5 % dry milk in 1 x Maleic Buffer) for 1 h and then incubated with anti DIG antibody at 1: 10,000 dilution for 30 min. The membrane was washed 2 x 100 ml with Washing Buffer and then equilibrated with Detection Buffer for 3 min, then with 1 ml CDP-Star for 5 min in a sheet protector followed by exposure to X-ray film for 15 min, then 5 min, then 1 min (3 films). The films were developed in a dark room. The molecular marker and probe control lit up and

only two PCR bands were lit up. Remove the bands from a fresh DNA gel and perform cloning and sequencing. However, before the Southern method was worked out, *dar1* 3' UTR was successfully cloned thus the method was not used. The successful 3' RACE for *dar1* cloning was described in chapter III.

5. 4 Mouse Kidney D-aspartate Oxidase Cloning, Expression and Application

An alternative method to analyze D-aspartate racemase reaction results is to use a color assay, quite distinct from the CE-LIF assay described previously. The color assay can be achieved by using D-aspartate oxidase (DAO). The enzyme can degrade D-Asp to oxaloacetate, ammonia and hydrogen peroxide in the presence of flavin adenine dinucleotide (FAD), a redox cofactor. Oxaloacetate is α -keto acid which can react with 2, 4-dinitrophenylhydrazine (DNPH) to form a brown colored complex 2,4-dinitrophenylhydrazone (DNPHn) in the presence of NaOH. The DNPHn can absorb light at 445 nm and the absorbance has linear relationship with D-Asp concentration within the range of 0-1.5 mM. The color assay has a few advantages compared to CE-LIF. For most, as many as 96 samples can be assayed simultaneously versus one sample a time with CE-LIF. The assay is compatible with TCA (enzyme assay stopper reagent) which has to be removed for CE-LIF. No amino acid derivatization is required which is essential for CE-LIF analysis. The color assay also allows various controls to run simultaneously. The goal of the project is to clone DAO from mouse kidney and make DAO reagent for D-Asp assay.

DAO was cloned by RT-PCR technique, expressed in *E. coli* and purified with NiNTA affinity resin as described below. DAO gene specific PCR cloning primers were designed based on the published

cDNA sequence by Katane (1). To clone the cDNA mouse kidney cortex was quickly dissected from a sacrificed animal. The tissue was quickly homogenized in 2 ml TRIzol (Invitrogen) reagent with motor-driven TissueRuptor (QIAGEN) homogenizer and total RNA was extracted from the homogenate by following TRIzol product instructions. Open reading frame (ORF) cloning was performed by RT-PCR method described as follows. Total RNA (1 µg) using Superscript III reverse transcriptase (Invitrogen) to make first strand cDNA with oligo dT primer following enzyme instruction. The first strand cDNA was then amplified by PCR using the following primers: 5'-GGCATATGGACACAGTGTGTATTGCGGT-3' (forward NdeI site underlined) and 5'-CCGATCCCTACAGCTTCGACAAGGAAGC -3' (reverse, Bam HI site underlined), and Platinum *pf*x DAN polymerase (Invitrogen) under the following thermo profile: 94 °C for 5min for 1 cycle; 94 °C for 15 sec, 55 °C for 30 sec and 68 °C for 80 sec for 29 cycles; 68 °C for 10 min. Target PCR fragment of about 1000 bp was removed and isolated from agarose gel with QIAGEN kit, ligated to pCR2.1®-TOPO® vector and transformed into TOP 10 chemical competent cells (Invitrogen). Ten blue/light blue colonies were picked from X-gal plate, grown up and plasmids were sequenced. The desired clone was grown up and plasmid isolated and digested with NdeI and Bam HI and ligated to bacterial protein expression vector pET15b and transformed into TOP10 cells. After sequence was confirmed, target clone plasmid was transformed into BL21(DE3) cells. The protein expression was performed by growing a 3 ml overnight bacterial starter culture in 250 ml LB at 30 °C until O.D. 600 equals 0.5. Protein expression was then started by adding 0.01 mM

IPTG to the culture and induced for 20 h at 30 °C. Recombinant muDAO was purified from bacterial lysate with NiNTA resin by following Chapter III DAR1 purification methods.

DAO activity was assayed by following the protocol as described by Katane et al.(1) with some modifications. To perform the enzyme assay, 40 mM D-aspartate substrate was incubated with 67 µM of FAD in 40 mM sodium phosphate buffer, pH 8.0 with 1 µg of purified enzyme in a total of 150 µl of reaction at 37 °C for 15 min. The reaction was then terminated by adding 5 % TCA followed by enzyme removal with centrifugation at 20, 000 x g for 10 min at 4 °C. The color assay was performed by adding 100 µl of 0.1 % 2, 4-dinitrophenylhadrozone in 2 N HCl and incubated at 37 °C for 15 min and then incubated with 750 µl of 3.75 M NaOH for 15 min followed by centrifugation at 8700 x g. The supernatants were read at O.D. 445 nm. The control samples had no amino acid substrate.

The protein was expressed in *E. coli* under 0.01 mM IPTG induction only and about 300 µg of purified protein was obtained from 250 ml bacterial liquid culture, no DAO was expressed without IPTG. The enzyme can oxidize D-Asp but not D-Ser. It also showed a weak activity to D-Glu. However, unlike pig kidney DAO which has a strong activity towards D-Glu. The mouse enzyme can be used to set up a quick assay for D-Asp production by racemase but sensitivity was not as good as CE-LIF. For DAR1 assay especially kinetics assay, very small amount of D-Asp was converted which can't be detected by DAO color assay. Thus the mouse DAO can be used for D-Asp racemase assay that is not quantitative.

5.5 Generation of Monoclonal Antibody against D-amino acid-containing Peptides

Like free D-amino acids, D-amino acid-containing peptides (DAACP) were thought to be only present in bacteria cell walls and antibiotics but not in higher organisms until after 1980 when some biological active DAACPs were found in frog skin (2), snail ganglion (3), snail venom (4), *Achitina fulica* and *Aplysia californica* ganglion (5) and Octopus brain (6), and Spider Venom (7). These peptides share a common feature of having a D-amino acid in the second position from the N-terminus with a few exceptions; the D-amino acids in the peptides can vary but include almost every category of amino acids - small, polar, charged and aromatic amino acids (8). The D isoform residues in these peptides confer their biological activities since the replacement of D-form with L-form amino acids diminish or abolish observed activities (9). Biological functions of these peptides are ranging from antimicrobial to cardioexcitatory to neuromodulation to hormonal functions. The DAACP biosynthesis is not very clear. But it could be from posttranslational modification (PTM) of L-amino acid configuration or incorporation of free-D-amino acid into a growing peptide during a ribosomal dependent or ribosomal independent biosynthesis process. But in eukaryotic system, predominant evidences have supported the conversion of L-amino acid residue to D-amino acid residue by isomerase after a peptide is translated (9, 10). So far two isomerases responsible for DAACPs biosynthesis have been obtained from spider venom (11) and frog skin (12). And most interestingly, an isomerase activity specifically in mouse heart tissue has been detected (13). The excitement of the field comes from the fact that most potent

cardioexcitatory peptides were found as DAACPs. For example, NdWFa (a D-amino acid was signified by a 'd' before a one code letter; 'a' at the end means C-terminus is amide) was found in several species of snails and shown potent cardioexcitatory activity based on pharmacological assay (14, 15). Because of their wide range and important biological functions, finding and studying D-amino acid-containing peptides are obviously important. The current approaches of identification and quantification of such peptides include analytical method such as HPLC (16), CE chiral separations (17), mass spectrometry (8), NMR (18), and biological methods such as ELISA (19), IHC (20), and enzymatic cleavage (21). Immunohistochemistry using antibody can offer a unique advantage over the other methods in that it can provide DAACPs spatial distributions at cellular and subcellular levels as a way to study their functions. But by the time when the project was started, there was no any monoclonal antibody (mAb) against DAACPs. Polyclonal antibody contains anti carrier protein antibodies besides anti DAACP specificity thus can produce ambiguous experimental results or high background staining for IHC experiments; but a mAb is of single specificity against a DAACP antigen and can be produced from an immortal hybridoma cell line thus have unlimited source. It is even better if a "class-specific" monoclonal antibody can be made. Such antibody can bind to any short peptides of 3-5 amino acids that contain D-amino acids at the second position from N-terminus. If it can be made, the class specific antibody would recognize D-amino acid backbone but not side-chain functional group of a D-amino acid in a peptide thus it allows the detection of any DAACPs in a tissue or cells by IHC method. The basic method to make a monoclonal antibody is to use animal immunization

followed by hybridoma fusion and screening of hybridoma. But to make the class specific monoclonal antibody, a special immunization strategy was used. That is to force immune system to select D-amino acid backbone by repeatedly exposing animals with the structure. Specifically, mice were immunized with three different groups of peptides, immunized with one group a time, once every three weeks until all three groups are immunized. Each group contains three different peptides and every peptide has a different D-amino acid at the second position at N-terminus. Such spatial structure specific mAb might be found from hundreds of thousands of hybridoma clones that recognize other parts of the antigens injected by extensive hybridoma screening with ELISA. An ELISA plate was coated with 9 different peptides. A screening hit should be able to recognize 9 peptides containing D-amino acids but not the control peptide set that contained only L-amino acids.

The following peptide antigens were used to immunize animals. Group I peptides were: YdAEFLCa, FdTEFLCa and GdFFDCa ; group II peptides were: NdWfa, AdLAAc and AdMAAc; group III peptides were: AdLAACa, AdRAACa and AdDAACa. In each group, three peptides were mixed at equal molar ratios before chemically linking them to carrier protein KLH which helps to stimulate immune response. Test peptides for ELISA include AdAAAa, AdAACa, VdVVCa, and VdVVa. The “a” signify amide and ‘c’ signify carboxylic acid. Group I and group III peptides are each conjugated to keyhole limpet hemocyanin (KLH) carrier protein (Calbiochem) using sulfo-SMCC (Pierce) cross-linker reagent by incubating 5-10 mg of carrier protein (KLH or BSA) with 2 mg of sulfo-SMCC in 0.1 M

NaPO₄ buffer, pH 7.2, containing 0.15 mM NaCl at RT for 1 h with gentle mixing. The activated KLH was purified through a home-made G-25 sephadex column and then mixed with 1 mg of a peptide to be conjugated and allowed to react at RT for 2 h with gentle stirring. The group II peptides were conjugated to KLH, individually, using cross-linker reagent 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) following the protocol by Hermanson (22). The different conjugation chemistries were chosen to avoid the same linker region between peptides and carrier protein KLH that are existed in all three groups thus might dominant immune response. The testing peptides were conjugated to BSA through either SMCC or EDC method. All conjugates were made at 1 mg/ml concentration based on carrier protein KLH or BSA quantity used.

The first step to the desired monoclonal antibody is to immunize animals (5 female Balb/c mice) with the three peptide groups which all contain D-amino acid backbones by i.p. injection. Group I conjugates were used for the primary immunization; group II for the secondary immunization and group III for the tertiary immunization. The adjuvant is to help antigen to stay in the injection sites so that immune cells can encounter them and to enhance immune response to immune cells. Thus the peptides were mixed with an oil based adjuvant to form semi-solid emulsion for immunization. An equal volume of antigen (containing 50-100 µg of antigen) and adjuvant was mixed and emulsified by sonication. A potent adjuvant was needed for primary immunization to prime animal immune systems, in this case, complete Freund's adjuvant was used to make antigen emulsion; and less potent incomplete

Freund's adjuvant (with less side-effect to animal) was used for the subsequent immunizations. The total antigen dosage was 50 µg in 100 µl antigen emulsion for one mouse (Balb/c mice, 8-10 weeks old when the first immunization was started). The second immunization was performed three weeks after the primary immunization and the tertiary immunization 3 weeks after the second one. One week from the third immunization, small quantity of blood was taken from each mouse by leg vein bleeding and each serum was tested against testing peptides in ELISA assay to see if the animals responded to the injected peptides.

The serum test results indicated that all five animals responded peptide antigens very well. Two best responders were sacrificed to make hybridoma cell lines. Each cell line can secrete a particular monoclonal antibody. The hybridoma cell lines were made by hybridoma technology described by Kohler et al. (23). To make hybridoma cell lines, a mouse (best immune responder) was sacrificed and the spleenocytes were isolated and fused with mouse myeloma cells with polyethylene glycol to produce fusion clones. About 200 fusion clones were grown up and their culture medium (containing monoclonal antibody) were screened against testing peptides in ELISA assay. The best clones with highest specific ELISA signal against DAACP were then tested against LAACP of the same peptide sequence.

Unfortunately, all positive clones that recognized DAACP also recognized its LAACP counterpart, meaning that the monoclonal antibodies were not specific to D-amino acid

backbone. The reason is likely due to that fact that peptide residue side chain functional groups have more chance to contact with immune cells than peptide backbones thus can stimulate immune systems. Since the side chain functional groups are the same in both DAACP and LAACP, immune system can't distinguish them thus the monoclonal antibodies were not specific to either peptide groups. Maybe the D-backbone specific antibody was there but it is certainly rare and requires an extensive screening to find it which means more time and cost would be involved. After considering the next steps it was decided that other approaches could be used to characterize DAACPs thus the project was dropped.

5.6 Microdialysis Fluid Analysis for L-Glutamate Change with CE-LIF

The goal of the project is to examine if L-glutamate injection at pedunculopontine tegmental nucleus (PPTg) at brainstem can stimulate L-glutamate change in rat hypothalamus Suprachiasmatic nucleus (SCN) at circadian clock 15 (or three hours after lights are turned off). The SCN is a part of the hypothalamus and controls circadian rhythms. This is a collaboration project with Dr. Martha Gillette's lab. My part of job was to analyze 72 microdialysis samples with CE-LIF and provide semi-quantitative data of L-Glu from each sample which was presented as L-Glu peak area normalized with CE-LIF internal control normalized L-cysteic acid peak.

SCN fluid samples are described in detail below. Nine mice received microdialysis device implants at hypothalamus SCN region. The mice were divided into three groups: A, B and C,

each group had three animals and received three different treatments at PPTg. From each group, microdialysis fluids from SCN were collected at 9 different time points. First, three baseline samples (before L-glutamate injection) were collected one hour before PPTg treatment with 20 min apart for each collection. The baseline samples were designated as B1, B2 and B3. At circadian clock 15 Group A received artificial cerebral spinal fluid; Group B received L-Glu (30 ng/nl) and Group C received three times higher concentration of L-Glu or 90 ng/nl by syringe injection at brainstem PPTg. SCN fluid samples were collected from each mouse at the time of injection and the samples were designated as INJ. Ten minutes after the injections, the first post-injection SCN fluid samples were collected from all animals, and then 20 min after injection and then 40 min and 60 min after injections. Thus there were 4 groups of post-injection samples that were designated as PS1, PS2, PS3 and PS4. In total, there are 27 baseline samples, 9 injection samples, 36 post-injection samples or 72 samples. Aliquots of 15 μ l SCN fluid from each sample was received from Martha Gillette Lab. The fluid samples were frozen at -80 °C immediately upon receiving until analyzed by CE-LIF.

The CE-LIF analysis of SCN fluid samples are described below. The samples were thawed and analyzed one a time. A fluid sample was slowly thawed on ice and 3 μ l was removed from the stock (remaining put back to -80 °C immediately) and derivatized with 1 μ l of 200 μ M of L-cysteic acid (L-CA) in H₂O, 3 μ l of 2 mM of NDA in methanol and 3 μ l of 20 mM KCN in 50 mM borate buffer, pH 9.4 at RT for 1 h. The sample was then diluted 1:1 with separation buffer containing 50 mM SDS, 20 mM β -cyclodextrin, 23 % methanol in 50 mM

borate buffer, pH 9.4. Derivatized sample was injected into CE capillary (50 μm inner diameter, 360 μm outer diameter) at 7 kV for 4 sec and separated under 20 kV for 45-60 min. Between two samples, the capillary was cleaned with 3 M NaOH for 5 min, rinsed with deionized water for 5 min and then equilibrated with the separation buffer until baseline was stabilized. The fluorescence counts of versus analyte retention time were plotted (automatically by the instrument software) to give rise to CE-electrophoregram. L-Glu, L-Asp and L-cysteic acid peaks were identified by standard amino acid spikes. L-Glu and L-Asp peaks were calculated by Origin8 integration function and the values were normalized with internal control L-CA peak area. The normalized analyte peak areas from three repeating mouse samples were averaged and presented in the figures.

The CE-LIF analysis results are shown in Figure 5.1 and Figure 5.2. Two representative CE-electrophoregrams from two sample runs were shown in Fig. 5.1 and normalized L-Glu and L-Asp values were shown in Figure 5.2. It is very interesting that L-Glu baseline samples showed more difference between animals than L-Asp baselines between the same animals, and in addition, the group of animals with high L-Glu baselines had lower L-Asp baselines and vice versa. During INJ, however, L-Glu was increased in all three groups but L-Asp was decrease in all three groups. After injection, L-Glu fell down quickly to the baseline level after twenty minutes but L-Asp continued to drop over 40 minutes of time and all the sudden showed a big increase after 60 min post-injection. Finally, the two dosages of L-Glu injection

with three times difference in L-Glu concentrations did not make any difference in SCN L-Glu and L-Asp response levels. Those data were used to plan following L-Glu experiments that are still undergoing.

5.7 Figures

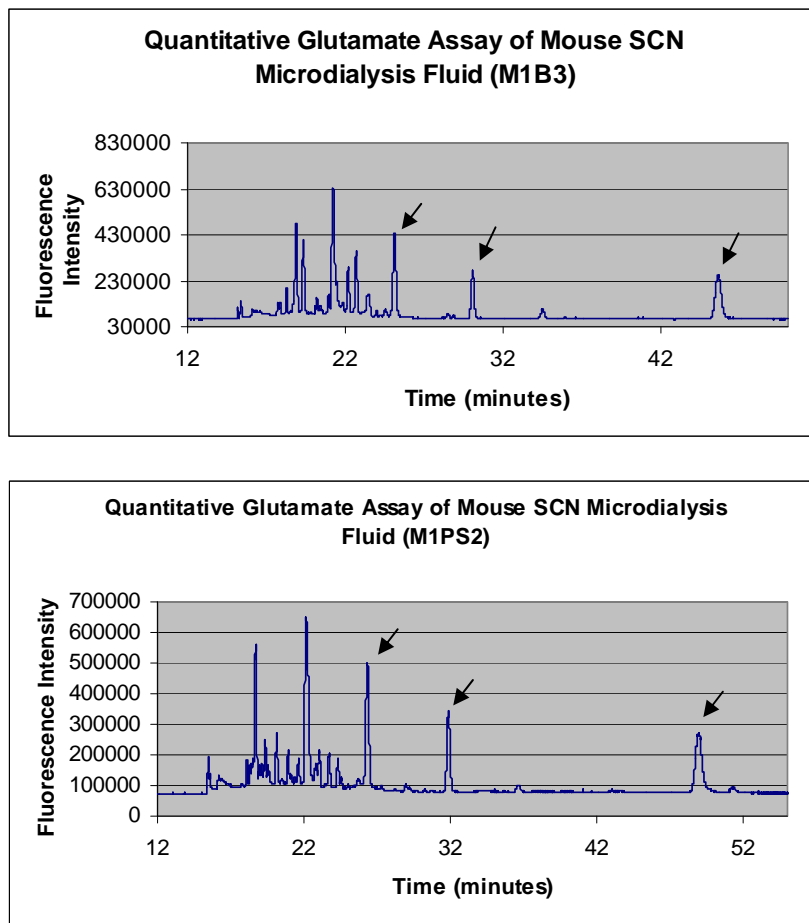


Fig. 5.1 CE-electrophoregram of mouse SCN microdialysis fluid. The experimental set up was described in section 5.5. Microdialysis fluid sample was collected from mouse #1 before L-glutamate injection to PPTg neurons (A). Microdialysis fluid sample was collected from mouse # 1 40 minutes after L-Glu injection to PPTg neurons (B). The peaks pointed by the left arrow are L-glutamate; by the middle arrow are L-Asp peaks and by the right arrow are L-cysteic acid.

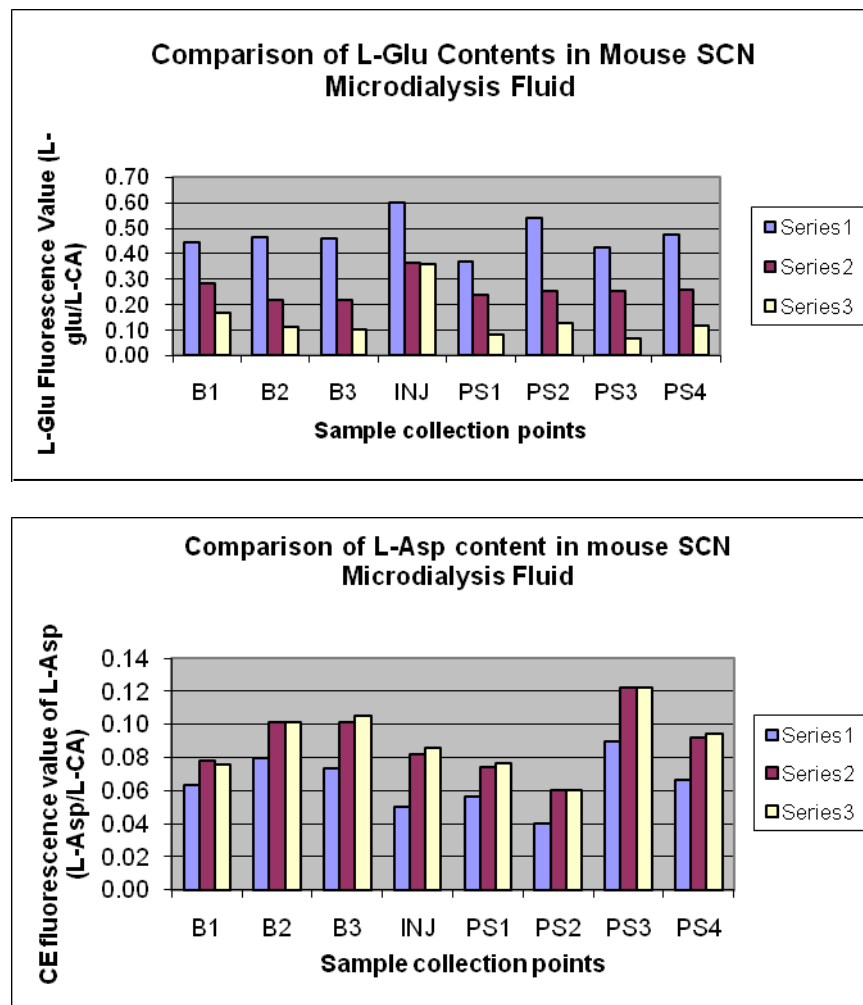


Fig. 5.2 Mouse SNC microdialysis fluid L-Glu and L-Asp content analysis by CE-LIF. L-Glu analysis data (A); L-Asp analysis data (B). B1-B3 are baseline samples collected before PPTg stimulation with L-glutamate, INJ samples were collected at injection time, PS1-PS4 samples were collected 10 min, 20 min, 40 min and 60 min after the injection time. Each bar represents average relative fluorescence counts adjusted by internal control from three mice received the same treatment. Series 1 were Group A samples, series 2 were Group B samples and series 3 were Group C samples. The treatment of each group was described in the text in section 5.5.

5.8 References

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