Identification and characterization of D-amino acid aminotransferase mutants using oxidase-coupled assays

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the MSc degree in Chemistry

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Abstract:

D-Amino acid aminotransferase (DAAT) is a pyridoxal-dependent enzyme that catalyzes the formation of a variety of D-amino acids, making it an attractive biocatalyst for the production of enantiopure D-amino acids. To bolster its biocatalytic applicability, improved variants displaying increased activity towards non-native substrates are desired.

In this work, we first developed a high-throughput, colorimetric, continuous coupled enzyme assay for the screening of DAAT mutant libraries that is based on the use of D-amino acid oxidase (DAAO). In this assay, the D-amino acid product of DAAT is oxidized by DAAO with concomitant release of hydrogen peroxide, which is detected colorimetrically by the addition of horseradish peroxidase and *o*-dianisidine. Using this assay, we identified three DAAT mutants (V33D, V33G and V33Y) displaying altered substrate specificity via the screening of cell lysates in 96-well plates. More specifically, the V33G and V33Y mutants display a ≈3-fold increase in k_{cat}/K_M for the non-native acceptor phenylpyruvate over wild type. Furthermore, we developed a coupled enzyme assay for the activity of DAAT with almost any D-amino acid using D-aspartate oxidase (DDO) as a coupling enzyme. Using this assay, we measured the specific activity of wild-type DAAT and the V33D, V33G, and V33Y mutants with 16 D-amino acid substrates. We demonstrate that the V33D, V33G, and V33Y mutants are more active than wild type with the aromatic substrates D-phenylalanine and 3-methyl-D-phenylalanine, while V33D and V33G are more active than wild type with D-valine, a branched chain amino acid. We expect the assays developed herein and subsequent mutants discovered to serve as valuable tools in the further development of biocatalysts for the production of enantiopure D-amino acids.

Acknowledgements:

First and foremost, I would like to thank my supervisor, Dr Roberto Chica, for his continued patience and belief in my abilities as a scientist. While we both know that I was not always the ideal grad student, I know that I would not have made it to this point without the pat on the back and the kick in the butt that you were able to provide. I have learned a lot about the scientific method, and especially the writing and presenting of results. I know that these tools will serve me well in my future studies of medicine.

Secondly, I would like to thank the current and past members of the Chica Lab for providing a fun and friendly work environment. Thank you especially to Curtis Walton, who walked me through numerous molecular biology techniques and was always available to troubleshoot my problems. You guys all made grad school that much better.

Thirdly, thank you to my amazing and supportive boyfriend, Eric Lortie, for waiting for me on late nights in the lab, hanging out with me when I had to come in on weekends, and generally pushing me to be better. You've seen me through these two years!

Finally, thank you to the NSERC Create program and the University of Ottawa for funding my studies. I'd also like to add a last shout-out to the Starbucks at the corner of Ogilvie and Bathgate for letting me pretty much live there while I wrote this whole thing. I appreciated the occasional discounts and the friendly distractions from writing!

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CE-MS, capillary electrophoresis coupled with mass spectrometry; DAAT, D-amino acid aminotransferase; DAAO, D-amino acid oxidase; DDO, D-aspartate oxidase; FAD, flavin adenine dinucleotide; FDH, formate dehydrogenase; GDH, L-glutamate dehydrogenase; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; L-AAD, L-amino acid deaminase; LAAO, L-amino acid oxidase; LDH, lactate dehydrogenase; NADH, β-nicotinamide adenine dinucleotide; NAD^+, β-nicotinamide adenine; NMDAr, *N*-methyl-D-aspartate receptor; PCR, polymerase chain reaction; PLP, pyridoxal 5'-phosphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Contribution Statement:

Conception: The direction of the project was determined by my supervisor, and we collaborated on the design of experiments.

Writing: Chapters 1, 3, and 4 were written by me and edited by my supervisor. Chapter 2 was co-written by my supervisor and me.

Experimental: All of the data shown in figures and tables was obtained through experiments I performed myself. For Chapter 2, the contributions of the authors besides my supervisor and me are as follows: Adam M. Damry constructed the pETDuet-DAAO-null plasmid; Guido F. Calderini prepared the pET11a-Histag-DAAO plasmid; and Curtis J.W. Walton prepared the DAAT-Y31X library construct.

1. Introduction

1.1 D-amino acids in nature

Amino acids are found in all forms of life as they are the building blocks of all proteins. Amino acids can also act in other capacities in living systems, such as in metabolism and in signalling. With the exception of glycine, all proteinogenic amino acids exist as two stereoisomers referred to as L and D. While L-amino acids represent the overwhelming majority of amino acids found in nature, D-amino acids are present as well, and serve many important functions in both prokaryotic and eukaryotic organisms.

Most naturally occurring D-amino acids are produced by bacteria, and consequently D-amino acids are often found in soil, fermented foods, and feces [1]. The most important function that D-amino acids serve in bacteria is their incorporation into the cell wall peptidoglycan (Figure 1.1A). Peptidoglycan is a polymer consisting of peptides ranging from three to five amino acids in length and the sugars *N*-acetylglucosamine and *N*-acetylmuramic acid. The peptide chains can be cross-linked to other peptides, forming a mesh-like structure that is the cell wall. The incorporation of D-amino acids into the peptidoglycan is fairly conserved: D-glutamate is usually the second residue in the peptide [2], while D-alanine occupies the fourth position and usually the fifth as well. In certain vancomycin-resistant bacteria, D-serine is added at the fifth position [3]. Some recent studies have shown that other D-amino acids (namely D-methionine and D-leucine) are incorporated into peptidoglycan by a diversity of stationary phase bacteria [4, 5]. It was further shown that genetic factors controlling the incorporation of these amino acids that are active during stationary phase can also be activated in response to stress, indicating that D-amino acids may aid bacteria in responding and adapting to various environmental strains [5].

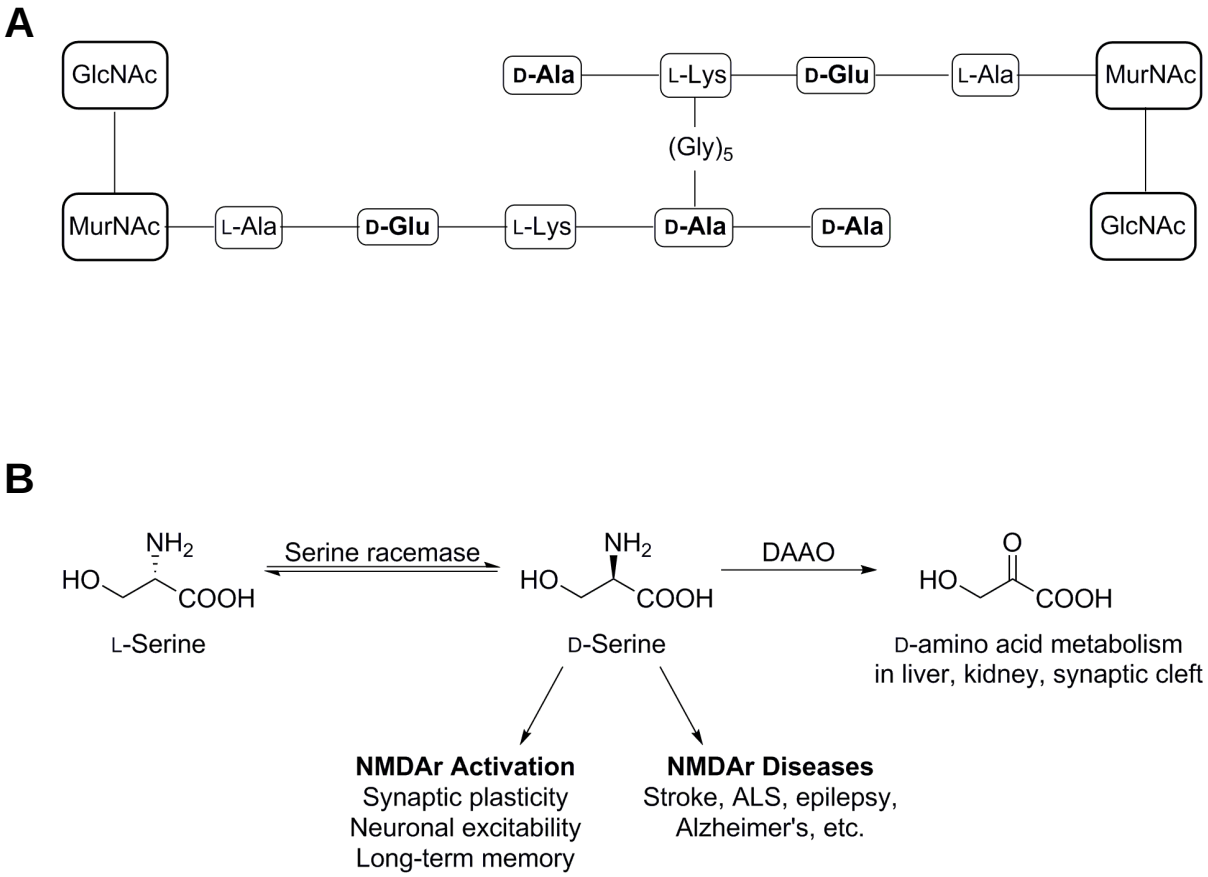


Figure 1.1 Examples of D-amino acid functions in biological systems. (A) D-glutamate and D-alanine are important components of the bacterial peptidoglycan which acts as a cell wall. GlcNAc is *N*-acetylglucosamine and MurNAc is *N*-acetylmuramic acid. (B) D-serine is produced in the central nervous system of vertebrates and acts as an agonist to the *N*-methyl-D-aspartate receptor (NMDAr).

The biosynthesis of D-amino acids in bacteria is accomplished using racemases, epimerases, and aminotransferases [1]. Most commonly, D-amino acids are synthesized through enzymatic racemization of their L-isomers. Racemases can be cofactor-dependent or independent (the cofactor being pyridoxal 5'-phosphate, or PLP), have narrow substrate specificity, and catalyze entirely reversible reactions. D-Alanine, D-glutamate, and D-aspartate are the only D-amino acids that are known to be synthesized by aminotransferases in nature (they are synthesized by racemases as well), and these enzymes are only found in certain bacteria, mainly bacilli. Aminotransferases have broader substrate specificity than racemases, making them attractive biocatalysts for the production of enantiopure D-amino acids.

In eukaryotes, it was widely believed that D-amino acids served no function, and were only acquired in these systems through diet, micro-organisms in the gut, or spontaneous racemization of L-amino acids in proteins through ageing [6]. Over the last few decades, it was shown that not only are D-amino acids present in significant concentrations in mammals, they also serve very important functions in the central nervous system. D-Serine is a co-agonist of the *N*-methyl-D-aspartate receptor (NMDAr, Figure 1.1B), and plays an important role in long-term synaptic plasticity of neurons [7], with regulation of its concentration modulating the excitability of neurons [8]. This amino acid has also been implicated in disease states associated with the NMDAr, which include stroke, epilepsy, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, and Huntington disease [9]. It is synthesized from L-serine by a serine racemase in the tissues where it is required, and metabolized by D-amino acid oxidase (DAAO). Another D-amino acid, D-aspartate, binds NMDAr as well but its function is related to maturation and differentiation of tissues where it

transiently appears [10, 11, 12]. D-Aspartate is synthesized from L-aspartate via an aspartate racemase [13] and is metabolized by D-aspartate oxidase (DDO) [14]. Additionally, high levels of D-serine, D-alanine, D-proline, D-asparagine, and D-aspartate in blood have been associated with ageing and renal disease [15]. It is suggested that the purpose of DAAO and DDO, found in high concentrations in the liver and the kidneys, is to metabolize these compounds as to prevent a toxic build-up [14]. Therefore, while L-amino acids were thought for decades to be the only form of amino acids that served biological functions, it is clear that D-amino acids are essential to life as we know it.

1.2 D-Amino acids in the pharmaceutical industry

In addition to playing important functional roles in both prokaryotes and eukaryotes, D-amino acids are finding increasingly numerous applications in the pharmaceutical industry. D-Amino acid moieties are present in many naturally occurring antibiotics, such as penicillins, and are also important building blocks for the synthesis of peptide-based drugs. Incorporation of D-amino acids in synthetic peptides is increasing due to their improved resistance to proteolytic degradation. In addition, D-amino acids are also being used clinically to tackle hearing loss [16].

A high number of natural products contain D-amino acid moieties. Antibiotics such as vancomycin (D-valine), penicillin (D-valine), cephalosporin C (D-homoglutamate), and gramicidins (D-phenylalanine) (Figure 1.2) all contain D-amino acids in their structures [17]. Semi-synthetic antibiotics such as amoxicillin (Figure 1.2) and ampicillin contain *para*-hydroxy-D-phenylglycine and D-phenylglycine, respectively [18]. Based on their presence in

so many naturally-occurring and semi-synthetic antibiotics, it is likely that D-amino acids will continue to be incorporated into new antibiotics as they are developed.

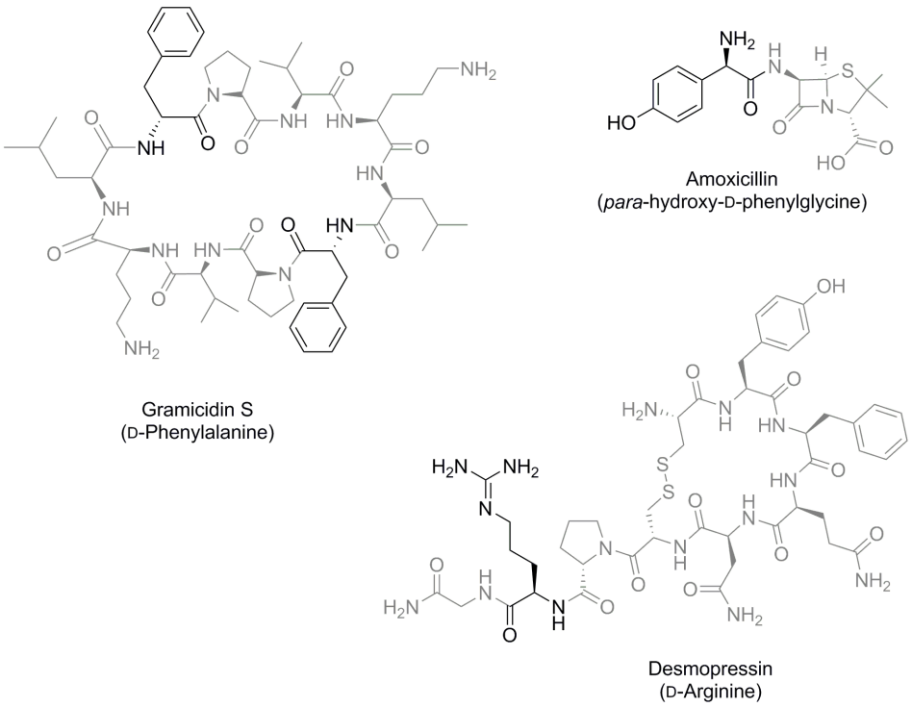


Figure 1.2 Examples of D-amino acids in pharmaceuticals. D-amino acid moieties are present in a variety of peptide-based pharmaceuticals, such as antibiotics and hormone mimics. Drug structures are shown in grey, with the D-amino acid they contain drawn in black for clarity.

Peptide-based drugs are an area of significant growth in the pharmaceutical industry, due in part to the discovery that incorporation of D- and β-amino acids helps to stabilize the peptide *in vivo*. For example, it was shown that apolipoprotein A-I mimetic peptides (for the treatment of atherosclerosis) synthesized from D-amino acids remained stable and functional in circulation in mice, while the L-form was rapidly degraded [19]. However, the use of

peptide analogs made from D-amino acids as hormone mimics is not a new concept: the drug desmopressin (Figure 1.2), a vasopressin mimic which contains D-arginine, has been on the market since the early 1970s [20]; the gonadotropin-releasing hormone agonist goserelin, which contains D-serine, has been researched extensively in cancer therapies for over 30 years; and the somatostatin peptide analog octreotide, which contains D-phenylalanine and D-tryptophan has been instrumental in both the study of the role of somatostatin in the body and the treatment of tumours [21]. These examples represent only a minute fraction of the drugs available on the market today that contain D-amino acids. The high resistance to protease degradation is also important for the design of drug delivery systems, and one such hydrogel based on D-amino acid dipeptides has been developed and shown to resist proteinase K hydrolysis, and efficiently accomplish a controlled drug release *in vivo* [22]. Because of these advantages, the use of D-amino acids in peptides for clinical purposes is expected to grow in the decades to come.

The use of free D-amino acids in clinical treatments is also being investigated. It has been shown that aural application of a D-methionine solution inhibits hearing loss induced by drugs such as cisplatin or carboplatin [16]. There is currently a Phase 3 clinical trial underway as well at Southern Illinois University to prove the efficacy of D-methionine in preventing noise-induced hearing loss (NCT01345474). Due to its presence in the central nervous system, potential therapeutic effects of D-serine are also being investigated [23]. The use of free D-amino acids as clinical treatments is still in its infancy, and there is no telling whether more applications will be discovered in the future.

Overall, D-amino acids are compounds that are of great value to the pharmaceutical industry. Their use in peptide-based drugs is especially important due to the increased

stability of these drugs *in vivo*. Therefore, it is essential that efficient and low-cost methods are developed for their synthesis.

1.3 Biocatalysis for the production of enantiopure D-amino acids

The use of enzymes for the production of optically pure chiral compounds is attractive due to the high enantioselectivity of many enzymes, and as such biocatalytic processes have been extensively developed over the last few decades. The use of enzymes for the production of enantiopure L-amino acids was first reported using acylases, and in fact the resolution of *N*-acetyl DL-methionine using acylases is the continued method of choice for the production of L-methionine [24]. Most of the methods for the biocatalytic preparation of amino acids described in the literature are focused on L-amino acids. The difficulty in applying these methods to the production of D-amino acids is that the enzymes used for the preparation of L-amino acids are highly stereospecific, and in many cases analogous enzymes for the corresponding D-amino acids are either unknown or have properties that hinder their application as biocatalysts. Nonetheless, biocatalysis for the production of D-amino acids is feasible, and enzymes including acylases, hydantoinases, dehydrogenases, oxidases, and aminotransferases are used for this purpose.

The use of stereospecific amino acid acylases for the deacylation of racemic *N*-acyl amino acids represents one of the first enzymatic methods for producing optically pure amino acids. A commercial process that is currently used for the resolution of compounds such as D-methionine, D-leucine, and D-phenylalanine exploits *N*-acyl-D-amino acid amidohydrolase (more simply referred to as D-aminoacylase) which deacylates neutral *N*-acyl D-amino acid derivatives [25] (Figure 1.3A). In this process, a racemic mixture of an

amino acid is acetylated by acetic anhydride, and then the *N*-acetyl-D-amino acid is deacylated, leaving the *N*-acetyl-L-amino acid untouched, and producing the corresponding D-amino acid. The *N*-acetyl-L-amino acid is recycled back to the racemic form via chemical racemization, and the cycle continues. D-glutamate and D-aspartate can also be produced by this method using *N*-acyl-D-glutamic acid amidohydrolase and *N*-acyl-D-aspartic acid amidohydrolase, respectively. A similar process is used for the production of *N*-benzyloxycarbonyl-D-proline, used in the synthesis of Eletriptan, a drug used to treat migraines. In this method, an L-proline acylase from *Arthrobacter* sp. is used to resolve *N*-benzyloxycarbonyl-DL-proline [18]. In this case, deacylation does not produce the desired amino acid, but rather degrades the unwanted enantiomer.

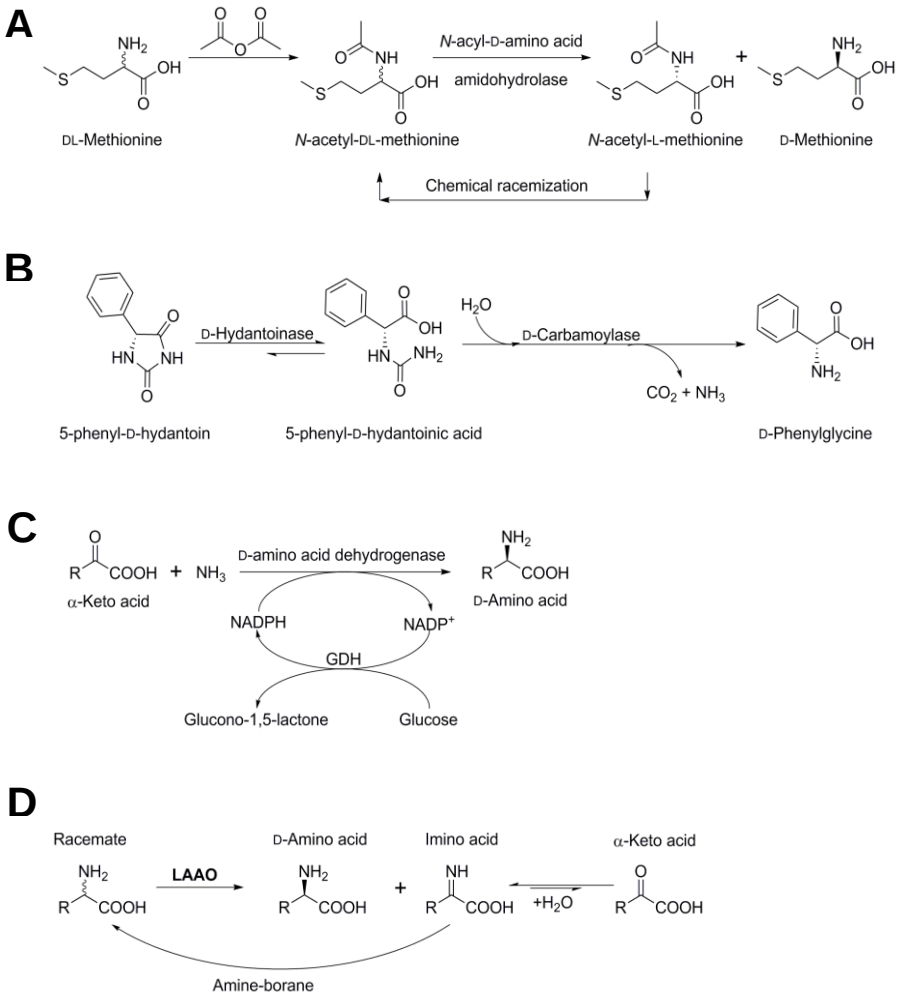


Figure 1.3 Biocatalytic methods for the synthesis of D-amino acids. A) Synthesis of D-methionine starting from the racemate using *N*-acyl-D-amino acid amidohydrolase. B) Synthesis of D-phenylglycine starting from the corresponding D-hydantoin, using D-hydantoinase and D-carbamoylase. C) General scheme for synthesis of D-amino acids using a D-amino acid dehydrogenase to catalyze the reductive amination of an α-keto acid. GDH is glucose dehydrogenase, used for cofactor regeneration. D) General scheme for the deracemization of amino acids to produce enantiopure D-amino acids using L-amino acid oxidase (LAAO) and amine-boranes.

Synthesis using D-hydantoins as a starting material and a system of hydantoinases and *N*-carbomoylases is used to produce D-phenylglycine (Figure 1.3B) and *p*-hydroxy-D-phenylglycine, compounds used in the synthesis of ampicillin and amoxicillin, respectively. Other amino acids that have been produced industrially by this method include D-leucine, D-allo-isoleucine, D-serine, D-allo-threonine, D-phenylalanine, D-histidine, D-glutamate, and D-lysine, among others [18]. This conversion is accomplished using racemic 5-monosubstituted hydantoin compounds, and a D-hydantoinase which cleaves the hydantoin ring to produce the resulting D-*N*-carbamoyl amino acid. A D-*N*-carbamoylase catalyzes further hydrolysis to the appropriate D-amino acid. Enzymes that degrade hydantoins are well-documented, and the first crystal structure of an *N*-D-carbamoylase from *Agrobacterium* sp. has been elucidated [26]. Directed evolution approaches have been used to engineer an improved hydantoinase variant for the production of L-methionine [27], therefore similar approaches could potentially be engaged for the production of D-amino acids as well. A fusion protein of a D-hydantoinase and *N*-D-carbamoylase has also been created [28]. This type of catalyst is advantageous in industry, as it would require the expression and purification of only one protein. Overall, the hydantoinase process is favoured for the synthesis of certain amino acids as it allows for close to 100% product conversion and 100% optical purity.

Amino acid dehydrogenases are currently employed in the synthesis of many L-amino acids [29]. These enzymes are useful as they allow the synthesis of enantiopure amino acids through reductive amination from the corresponding α-keto acid and ammonia (Figure 1.3C). Recently, the creation of a D-amino acid dehydrogenase with broad substrate specificity was reported [30]. The enzyme was created through directed evolution and rational design of *meso*-2,6-D-diaminopimelic acid dehydrogenase, and requires the cofactor

NADPH. The engineered enzyme was shown to catalyze the reductive amination of a number of α-keto acids to produce D-amino acids with high enantiomeric excess, using glucose dehydrogenase for cofactor regeneration. However, the specific activity of the enzyme towards the substrates tested was quite low. Further engineering to enhance activity would be needed in order to make this enzyme viable for industrial biocatalytic applications.

Amino acid oxidases have been employed for the resolution of racemic mixtures of amino acids. Specifically, D-amino acid oxidase (DAAO) has been used to deracemize L-amino acids, as DAAO selectively deaminates D-amino acids to their corresponding α-keto acid. An example is a method developed for the resolution of L-proline, in which D-proline is oxidized by DAAO, followed by reduction by sodium borohydride to produce racemic proline [31]. The cycle continues until only L-proline remains, and the authors report that no D-proline was detected via enantioselective HPLC. The authors comment that this system could theoretically be applied for the production of D-amino acids from a racemic mixture, given that an appropriate L-amino acid oxidase (LAAO) is available. One such LAAO from *Rhodococcus* sp. AIU Z-35-1 is described that possesses broad substrate specificity and was demonstrated to resolve D-glutamine, D-homoserine, and D-arginine, which are not produced by acylases or hydantoinases [32]. Further research is needed to develop a process using this enzyme. Another LAAO from *Proteus myxofaciens* has been incorporated into a scheme similar to that described above by Soda *et al*, using amine-boranes as a reducing agent (Figure 1.3D) [33]. This method was able to produce a range of D-amino acids starting from the racemate, including D-norvaline, D-tryptophan, D-phenylalanine and D-cyclopentylserine in yields above 80% and with >99% enantiomeric excess. The drawback to deracemization using amino acid oxidases is that the imino compound must be stable enough to be recycled

back to the amino acid form. Therefore, some amino acids cannot be produced using these methods, as their imino form will quickly oxidize to the keto form. Thus, deracemization methods using amino acid oxidases are attractive choices for obtaining optically pure amino acids, provided that the racemic starting material can be regenerated in multiple rounds of deracemization.

Finally, aminotransferases are used in the synthesis of D-amino acids. They can synthesize D-amino acids from the corresponding α-keto acid using a D-amino acid donor, and their use in the enzymatic production of these compounds will be discussed in detail in the next section.

1.4 D-amino acid aminotransferase as a biocatalyst for the production of enantiopure D-amino acids

Aminotransferases (EC 2.6.1.X) (also called transaminases) are pyridoxal 5'-phosphate (PLP) dependent enzymes that catalyze the transfer of an amino group from an amino acid to an α-keto acid to generate a new amino acid/α-keto acid pair. L-α-amino acid aminotransferases are ubiquitous in nature as they are the principal enzymes responsible for amino acid biosynthesis; however, D-amino acid aminotransferases have been identified in many bacterial species. Aminotransferases possess many qualities that make them ideal for use as biocatalysts in the asymmetric synthesis of amino acids: They have very high enantioselectivity, broad substrate specificity, high reaction rates, and no need for external cofactor regeneration [34]. Indeed, biocatalytic processes involving aminotransferases have been developed, and engineering improved aminotransferase variants remains an important research goal.

The transamination reaction catalyzed by aminotransferases proceeds through a bi bi ping-pong mechanism, and is well characterized. The first half-reaction involves the formation of a Schiff base between the amino acid substrate and PLP, replacing the Schiff base between a catalytic lysine residue and PLP, forming the external aldimine. The catalytic lysine residue then acts as a base for proton transfer from the α-carbon of the substrate to the cofactor, forming the ketimine intermediate. This intermediate is then hydrolyzed, releasing an α-keto acid and forming a pyridoxamine 5'-phosphate [35]. The second half-reaction involves reversal of these steps initiated through binding of an α-keto acid to release an amino acid. Aminotransferase-catalyzed reactions predictably have an equilibrium constant close to unity.

D-Amino acid aminotransferase (DAAT, EC 2.6.1.21) is involved in the biosynthesis of D-glutamate which is an important component of the bacterial cell wall peptidoglycan [36]. It catalyzes the transamination of α-ketoglutarate with D-alanine, producing D-glutamate and pyruvate (Figure 1.4), but is active with other D-amino acids as well. In fact, DAAT has even been shown to use L-amino acids as substrates, although in these cases the products of the enzyme reaction are exclusively D-amino acids [37]. Overall, DAAT reacts most efficiently with D-alanine, D-glutamate, D-aspartate, D-methionine, D-glutamine, and D-asparagine, and is a poor catalyst for the transamination of aromatic and branched chain D-amino acids.

Figure 1.4 Transamination reaction catalyzed by DAAT. DAAT catalyzes the reversible transamination of pyruvate with D-glutamate to produce α-ketoglutarate and D-alanine.

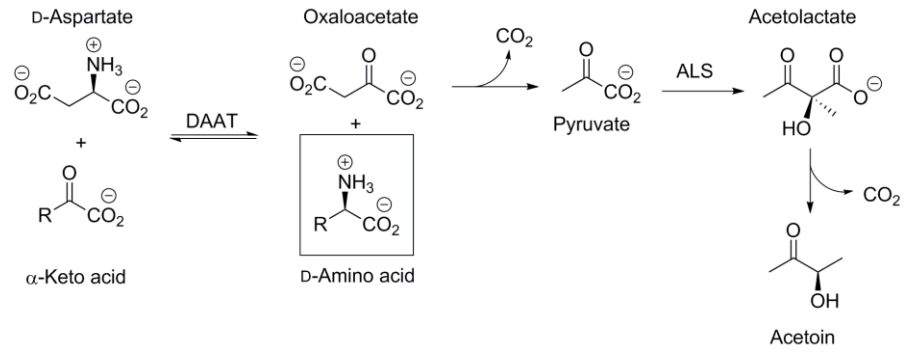
There are many published methods of synthesizing D-amino acids using DAAT from either *Bacillus sp.* YM-1 or *Bacillus sphaericus*, all of which are multi-enzyme systems. Issues that have been faced in developing these processes include driving the equilibrium towards product, acquiring a D-amino acid donor, and acquiring an appropriate α-keto acid acceptor. D-Amino acids are often expensive or unavailable commercially (hence the need for biocatalytic processes for their synthesis in the first place), and the same can be said of many α-keto acids. These issues have been addressed in the published methods through introduction of additional enzymes such as racemases and deaminases, and will be discussed thoroughly below.

One of the methods that addresses the issue of reaction equilibrium is a whole-cell process developed by Fotheringham *et al* [38]. In this method, genes for DAAT and acetolactate synthase (ALS) are transformed into the cells and expressed. D-Aspartate is used as an amino donor, while many α-keto acid can be used as the acceptor substrate. DAAT catalyzes the transamination reaction to form the D-amino acid product and oxaloacetate, which spontaneously decarboxylates to form pyruvate (D-alanine could also be used as a donor; in this case pyruvate is produced directly via DAAT). ALS catalyzes the dimerization of pyruvate to form acetolactate, which decarboxylates spontaneously to form acetoin. This

prevents the transamination of pyruvate by endogenous aminotransferases, and drives the reaction towards the products. This method has been employed successfully for the production of D-glutamate and D-2-aminobutyrate; however, this method suffers from the drawback that both the D-amino acid and α-keto acid must be supplied externally, driving up costs.

Figure 1.5 DAAT as a biocatalyst for the production of enantiopure D-amino acids. (A) The DAAT-catalyzed transamination of an α-keto acid with D-aspartate can be coupled with acetolactate synthase (ALS) to drive the equilibrium towards the formation of a new D-amino acid product in a whole-cell process. (B) The D-alanine donor for the transamination reaction is synthesized from L-alanine using alanine racemase (AlaR). Then, DAAT catalyzes the transamination of an α-keto acid with D-alanine to produce the desired D-amino acid and pyruvate. Pyruvate is recycled back to D-alanine through L-alanine dehydrogenase (L-ADH) and AlaR. The NADH cofactor of L-ADH is regenerated using formate dehydrogenase (FDH). (C) An α-keto acid is produced through deamination of an L-amino acid catalyzed by L-amino acid deaminase (L-AAD), while D-glutamate is produced via the racemization of L-glutamate by glutamate racemase (GluR). DAAT catalyzes the transamination reaction to form a D-amino acid product and α-ketoglutarate, the latter being is recycled back to L-glutamate via endogenous L-amino acid aminotransferases (LAAT) or catabolized by the cell, driving the reaction towards the products. (D) D-Phenylalanine is produced in cells lacking the genes necessary to produce L-phenylalanine through transamination. Endogenous D-alanine acts as an amino donor to endogenous phenylpyruvate, and DAAT catalyzes the transamination reaction to produce pyruvate and D-phenylalanine. The production of D-phenylalanine is further enhanced by the mutation of D-alanine dehydrogenase (D-AlaDH) to prevent D-alanine degradation.

A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DAAT
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ALS
CO_2
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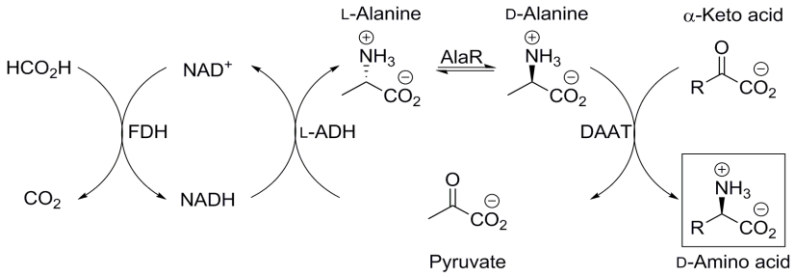
D-Amino acid
Acetoin

B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Pyruvate
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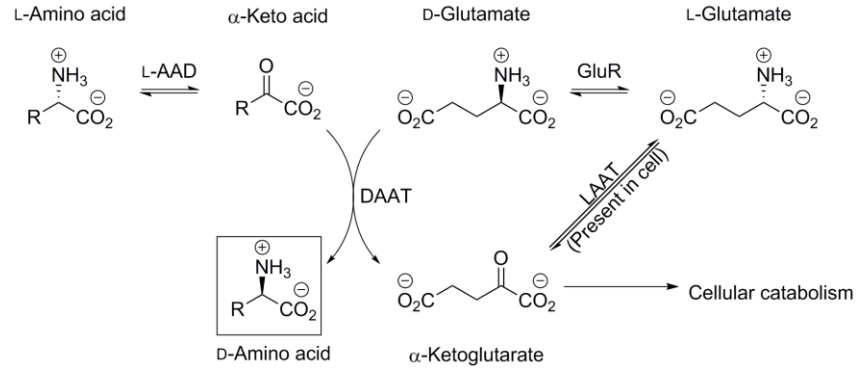
C


L-Amino acid
α-Keto acid
D-Glutamate
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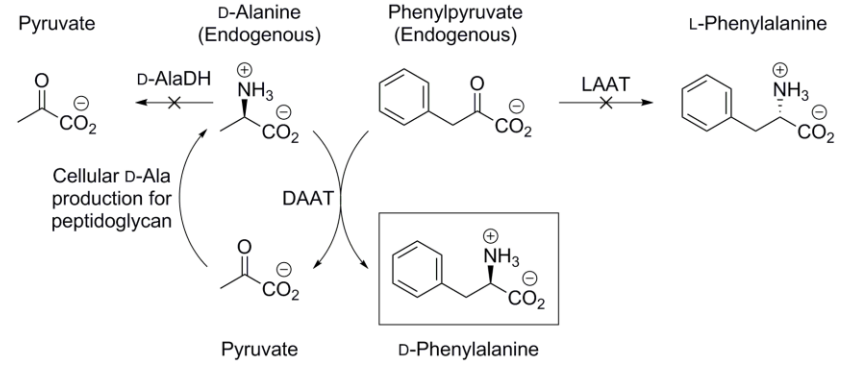
GluR
DAAT
LAAT
(Present in cell)
Cellular catabolism
D-Amino acid
α-Ketoglutarate

D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D-Alanine
(Endogenous)
Phenylpyruvate
(Endogenous)
L-Phenylalanine
D-AlaDH
LAAT
Cellular D-Ala
production for
peptidoglycan
DAAT
Pyruvate
D-Phenylalanine

A multi-enzyme reaction scheme that requires no external addition of D-amino acids has been described [39, 40, 41, 42]. In this method, D-glutamate or D-alanine is produced enzymatically *in situ* from α-ketoglutarate or pyruvate via L-glutamate dehydrogenase (GDH) and glutamate racemase, or L-alanine dehydrogenase and alanine racemase, respectively. DAAT then catalyzes the formation of a new D-amino acid through transamination of an added α-keto acid. The overall reaction is driven to completion as the α-ketoglutarate or pyruvate produced by DAAT is recycled back to D-glutamate or D-alanine via the dehydrogenases and racemases. Since the dehydrogenase enzymes require NADH as a cofactor, formate dehydrogenase (FDH) and formate are also included in the reaction to regenerate NADH. Using the alanine method [41], many D-amino acids were produced in high yield and high optical purity, including D-glutamate, D-leucine, D-norleucine, and D-methionine. D-Phenylalanine and D-tyrosine were produced with high optical purity as well, but the yields were significantly lower as these compounds are poor substrates of DAAT. Using the glutamate method [40] the yields of these aromatic products were increased to 100% when the concentration of the aromatic α-keto acids was kept below 50 mM. A drawback to these methods is that the expensive α-keto acids of the desired products are required, and the substrate concentrations need to be kept relatively low by industrial standards.

The most applicable method that theoretically addresses all of the issues mentioned has been developed by Fotheringham *et al* [43]. This is a whole-cell method in which the bacterial strain of choice has been transformed with recombinant DAAT, L-aminodeaminase from *P. myxofaciens* (L-AAD) [44], and appropriate amino acid racemase genes. In this method an L-amino acid, which is either fed to the cells or over-produced internally by

metabolic engineering, is converted to an α-keto acid acceptor by L-AAD. The D-amino acid donor is produced by feeding the cells L-glutamate, L-alanine, or L-aspartate which is converted to the D-isomer via the appropriate racemase. DAAT then catalyzes the formation of the desired D-amino acid product from the enzymatically-produced substrates. The overall reaction is shifted towards the products under fermentative conditions by cellular catabolism of the α-keto acid product of the transamination reaction and regeneration of the amino donor through cellular L-amino acid aminotransferases. Therefore, this method completely eliminates the need for feeding the reaction the most expensive components which are the D-amino acid donor and the α-keto acid acceptor. It also does not necessitate a cofactor regeneration system. Unfortunately, there is no published data describing the productivity of this system, but it can produce D-phenylalanine with 100% optical purity.

Finally, a method has been patented by the same authors for the production of D-phenylalanine using DAAT and host cell strains that do not express L-amino acid aminotransferases [45]. D-Alanine is produced endogenously by bacteria for cell wall synthesis, and phenylpyruvate is present in the cell as a precursor to L-phenylalanine. By using bacterial strains that lack the ability to produce L-phenylalanine through transamination, and expressing DAAT in the cell, D-phenylalanine can be produced exclusively. D-Phenylalanine production can be increased in this method by mutating the gene encoding D-alanine dehydrogenase, which contributes to D-alanine and D-phenylalanine degradation, rendering this enzyme inactive. It is enhanced further by expressing an alanine racemase to increase the amount of D-alanine substrate available to DAAT, and the *aroH* gene which deregulates the production of phenylpyruvate, increasing the availability of this substrate as well.

Overall, it is clear that DAAT is a useful biocatalyst for the production of enantiopure D-amino acids. Although DAAT has broad substrate specificity, it does react poorly with aromatic and branched chain D-amino acids. Therefore, the biocatalytic processes described above could be further improved through the development of DAAT variants with improved activity towards these and other non-native substrates. Since the crystal structure of DAAT is known [46], rational design coupled with high-throughput screening methods can be applied to generate mutants displaying altered substrate specificity that can be incorporated in the methods described above.

1.5 Scope of this work

In this study, we investigate the detection and characterization of DAAT mutants displaying altered substrate specificity through the development and implementation of oxidase-coupled assays. First, we develop a high-throughput, colorimetric assay based on the use of D-amino acid oxidase (DAAO) as a coupling enzyme. In this assay, the D-amino acid product of the DAAT-catalyzed reaction is oxidized by DAAO, producing hydrogen peroxide. The production of hydrogen peroxide is detected colorimetrically by the addition of horseradish peroxidase (HRP) and *o*-dianisidine. We use this assay to determine apparent kinetic parameters for purified DAAT, and detect the activity of DAAT mutants via the screening of clarified cell lysates. We show that this assay is more sensitive than the previously-developed L-glutamate dehydrogenase (GDH) coupled assay, as it was able to detect the activity of a mutant ≈2000-fold less active than wild type. Additionally, we use this assay to identify three mutants of DAAT (V33D, V33G, and V33Y) that are more active than the wild type with the non-native substrate phenylpyruvate. Of these mutants, DAAT V33G and

V33Y are shown to have k_{cat}/K_M values ≈3-fold higher than the wild type for phenylpyruvate. In order to further characterize the substrate specificity of the three identified mutants for their applicability as biocatalysts for the production of enantiopure D-amino acids, we develop another assay using D-aspartate oxidase (DDO) as a coupling enzyme. This assay allows us to detect DAAT activity with a wide range of D-amino acid donor substrates, as opposed to α-keto acid acceptors as is the case with the DAAO assay. Using this assay, we are able to profile the substrate specificity of wild-type DAAT and the V33D, V33G, and V33Y mutants with a wide range of D-amino acids, including some unnatural D-phenylalanine derivatives. Overall, this study presents the development of two novel coupled enzyme assays for the detection of DAAT activity, and identifies three mutants that are more active than the wild type with phenylpyruvate/D-phenylalanine. We expect that these assays and mutants will be important tools in the further development of DAAT as a biocatalyst for the production of enantiopure D-amino acids.

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2. Continuous Colorimetric Screening Assay for Detection of D-Amino Acid Aminotransferase Mutants Displaying Altered Substrate Specificity

Analytical Biochemistry 2014, Volume 463, pages 23-30

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Preface

In order to bolster the applicability of DAAT as a biocatalyst for the production of enantiopure D-amino acids, it is desirable to alter its substrate specificity to increase its activity toward non-native substrates. As part of an enzyme engineering strategy, it is necessary to have a screening method for the detection of mutants with desired activity. This method should be high-throughput, sensitive, and allow for the use of various substrates to probe altered substrate specificities. This chapter presents the development of high-throughput colorimetric coupled assay for the screening of DAAT mutant libraries using DAAO as a coupling enzyme. This work was published in *Analytical Biochemistry* in October 2014, and includes multiple authors besides myself. The contributions of the other authors to this study are as follows:

Adam M. Damry created the pETDuet-DAAO-null plasmid, and performed initial proof-of-principle experiments (not shown).

Guido F. Calderini created the pETDuet-DAAO-DAAT and the pET11a-Histag-DAAO plasmids.

Curtis J.W. Walton created the DAAT-Y31X library and performed the screening with the GDH coupled assay.

Roberto A. Chica was the principal investigator for this study, and as such designed experiments, analyzed results, and co-wrote the paper.

As primary author, Janet E.B. Barber designed experiments, performed experimental research, analyzed results, and co-wrote the paper.

Abstract

D-Amino acid aminotransferase (DAAT) catalyzes the synthesis of numerous D-amino acids, making it an attractive biocatalyst for the production of enantiopure D-amino acids. To bolster its biocatalytic applicability, improved variants displaying increased activity towards non-native substrates are desired. Herein, we report the development of a high-throughput, colorimetric, continuous coupled enzyme assay for the screening of DAAT mutant libraries that is based on the use of D-amino acid oxidase (DAAO). In this assay, the D-amino acid product of DAAT is oxidized by DAAO with concomitant release of hydrogen peroxide, which is detected colorimetrically by the addition of horseradish peroxidase and *o*-dianisidine. Using this assay, we measured apparent K_M and k_{cat} values for DAAT and identified mutants displaying altered substrate specificity via the screening of cell lysates in 96-well plates. The DAAO coupled assay is sensitive as it allowed the detection of a DAAT mutant displaying a ≈2,000-fold decrease in k_{cat}/K_M relative to wild type. Additionally, the DAAO assay enabled the identification of two DAAT mutants (V33Y and V33G) that are more efficient than wild type at transaminating the non-native acceptor phenylpyruvate. We expect that this assay will be useful for the engineering of additional mutants displaying increased activity towards non-native substrates.

Keywords: transaminases; coupled enzyme assay; high-throughput screening; mutagenesis; substrate specificity; enzyme kinetics

Abbreviations used: NADH, β-nicotinamide adenine dinucleotide, reduced dipotassium salt; NAD^+, β-nicotinamide adenine dinucleotide hydrate; GDH, L-glutamate dehydrogenase;

DAAT, D-amino acid aminotransferase; PCR, polymerase chain reaction; DAAO, D-amino acid oxidase; LDH, lactate dehydrogenase; HRP, horseradish peroxidase; FAD, flavin adenine dinucleotide disodium salt hydrate; PLP, pyridoxal 5'-phosphate monohydrate; IPTG, isopropyl β-D-1-thiogalactopyranoside.

2.1 Introduction

D-Amino acid aminotransferase (DAAT, EC 2.6.1.21) is a pyridoxal phosphate-dependent enzyme that is involved in the biosynthesis of D-glutamate, an important component of cell wall peptidoglycan in bacteria [1]. *In vivo*, it catalyzes the transfer of the amino group from donor substrate D-alanine to the α-ketoglutarate acceptor, producing pyruvate and D-glutamate. DAAT has also been shown to catalyze the synthesis of a broad range of D-amino acids *in vitro* [2; 3], making the enzyme an attractive biocatalyst for the production of enantiopure D-amino acids [4]. To further bolster the biocatalytic applicability of DAAT, improved variants displaying increased activity towards non-native substrates are desired.

In order to engineer improved DAAT variants, a sensitive high-throughput assay that can be used to screen mutant libraries with various candidate substrates is needed. Several assays have been developed to measure the activity and kinetic properties of DAAT. Many are discontinuous assays based on radiometric methods [5], chromatography [6] or capillary electrophoresis coupled with mass spectrometry [7]. While these assays are sensitive and allow for the detection of desired DAAT products, their application for the screening of large mutant libraries is impractical. An additional discontinuous assay in which the pyruvate product of DAAT is reacted with salicylaldehyde under alkaline conditions to yield an orange coloured compound has also been developed [3]. While this colorimetric assay could

be adapted for high-throughput screening of mutant libraries, it suffers from the drawback of requiring reaction quenching with concentrated base, a downstream sample processing step that can increase noise and introduce measurement errors.

Continuous assays have also been developed to measure DAAT activity. These assays are advantageous since they enable rates to be determined from a high number of data points, increasing precision. In these assays, the activity of DAAT is coupled to that of an additional enzyme that converts the DAAT product, yielding a compound that can be readily detected. Lactate dehydrogenase (LDH) [8] and L-glutamate dehydrogenase (GDH) [9] have both been used as coupling enzymes for measuring DAAT activity due to their ability to catalyze the reduction of the pyruvate or α-ketoglutarate products of the forward or reverse transamination reaction catalyzed by DAAT, respectively. Reduction of pyruvate/α-ketoglutarate by LDH/GDH is accompanied by the oxidation of NADH to NAD^+, which can be followed spectrophotometrically at 340 nm. While the GDH assay has been successfully applied to the high-throughput screening of DAAT mutant libraries [9], it has the limitation of measuring a signal decrease over time, which can reduce sensitivity for low turnover enzymes [10]. Sensitivity of the GDH screening assay is further decreased due to the high background signal at 340 nm caused by the presence of other molecules in cell lysates that absorb in the near-UV, including endogenous NADH. These same disadvantages would be expected when applying the LDH assay to the screening of DAAT activity in cell lysates. Therefore, there is still interest in developing a more sensitive high-throughput assay for the screening of DAAT mutant libraries.

In this study, we report the development of a high-throughput, colorimetric, continuous coupled enzyme assay for the screening of DAAT mutant libraries that is based

on the use of the flavoprotein D-amino acid oxidase (DAAO, EC 1.4.3.3), which has already been used as a coupling enzyme for measuring the activity and enantioselectivity of ω-aminotransferases [11]. In this assay, the D-amino acid product of DAAT is oxidized by DAAO with concomitant release of hydrogen peroxide, which can be detected colorimetrically by the addition of horseradish peroxidase and *o*-dianisidine. Using this DAAO coupled assay, we were able to measure apparent K_M and k_{cat} values for purified DAAT and identify DAAT mutants displaying altered substrate specificity via the screening of clarified cell lysates in 96-well plates. Our results demonstrate that the DAAO coupled assay developed herein is more sensitive than the GDH coupled assay [9] as it allowed us to detect the activity of a DAAT mutant displaying a ≈2,000-fold decrease in k_{cat}/K_M relative to the wild-type enzyme, a value ≈3-fold lower than that of the lowest activity mutant identified with the GDH assay. Additionally, use of the DAAO coupled assay enabled us to identify two DAAT mutants (V33Y and V33G) that display altered substrate specificity, being more efficient than the wild type at transaminating the non-native acceptor substrate phenylpyruvate.

2.2 Materials and Methods

2.2.1 Materials

All reagents used were of the highest available purity. Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs. Synthetic oligonucleotides were obtained from Integrated DNA Technologies, and Ni-NTA agarose resin was obtained from Promega. All aqueous solutions were prepared using water purified with a Barnstead

Nanopure Diamond system. Enzyme substrates, cofactors, horseradish peroxidase (HRP), and lysozyme were purchased from Sigma-Aldrich.

2.2.2 Preparation of plasmid constructs

A codon-optimized gene for *Trigonopsis variabilis* DAAO [12] obtained from GenScript was sub-cloned into multiple cloning site 1 of the pETDuet-1 vector (Novagen) via *Nco*I/*Hin*dIII, resulting in the pETDuet-DAAO-null plasmid. Then, the codon-optimized and His-tagged *Bacillus sp.* YM-1 DAAT gene contained in a pET11-a vector (Novagen) [7] was PCR-amplified using VentR DNA Polymerase (New England Biolabs) and sub-cloned into multiple cloning site 2 of pETDuet-DAAO-null via *Nde*I/*Aat*II, resulting in the pETDuet-DAAO-DAAT plasmid. An additional vector containing a His-tagged DAAO gene was also prepared to enable individual expression and purification of this enzyme. To do this, the DAAO gene from plasmid pETDuet-DAAO-null was PCR-amplified with VentR DNA polymerase using appropriate primers to append an *N*-terminal His-tag to the DAAO gene. The resulting amplicon was subsequently sub-cloned into pET11-a (Novagen) via *Nde*I/*Bam*HI. All plasmids were transformed into *E. coli* XL1-Blue cells (Stratagene) and the entire open reading frames were verified by sequencing.

2.2.3 Mutagenesis

Saturation mutagenesis was performed on the DAAT gene by overlap extension mutagenesis [13] using VentR DNA polymerase. Briefly, external primers were used in combination with sets of complementary pairs of oligonucleotides containing the NNS degenerate codon in individual PCRs. The resulting fragments were gel-extracted (Omega Biotek) and

recombined by overlap extension PCR. The resulting amplicons were introduced into the second multiple cloning site of pETDuet-DAAO-null via *Nde*I/*Aat*II, and all constructs were verified by sequencing the entire open reading frame.

2.2.4 Protein expression and purification

Proteins were expressed in 500 mL cultures (LB with 100 μg/mL ampicillin) of *E. coli* BL21-Gold(DE3) cells (Stratagene) transformed with either the pETDuet-DAAO-DAAT vector containing a mutant DAAT gene, or pET11a containing the wild-type DAAT or DAAO genes. Cultures expressing wild-type DAAT or one of its mutants were grown at 37 °C with shaking until an optical density of 0.6 at 600 nm was reached, while cultures expressing only DAAO were grown to an optical density of 1.0. At this point, protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells expressing DAAT or one of its mutants were incubated for 3 hours at 37 °C with shaking, while cells expressing only DAAO were incubated overnight at 17 °C with shaking. Following incubation, cells were harvested by centrifugation and lysed with an EmulsiFlex-B15 cell disruptor (Avestin). Proteins were purified by immobilized metal affinity chromatography according to the manufacturer's protocol. The eluted fractions were desalted by gel filtration using EconoPAC 10DG columns (Bio-Rad) into 100 mM potassium phosphate buffer (pH 8.0). Protein concentrations were quantified using a modified Bradford assay, where the calibration curve is constructed as a plot of the ratio of absorbance at 590 nm and 450 nm versus concentration [14].

2.2.5 Steady-state kinetics

One unit (U) is defined as the amount of enzyme that catalyzes the conversion of 1 μmole of substrate into product per minute. All kinetic assays were performed in triplicate 200-μL reactions at 37 °C in 100 mM potassium phosphate buffer (pH 8.0) and using enzyme from three independent preparations. For the determination of DAAO kinetic parameters, reaction mixtures consisted of appropriate amounts of the D-amino acid being studied (D-glutamate or D-alanine), 1 μM flavin adenine dinucleotide disodium salt hydrate (FAD), 0.5 mM *o*-dianisidine dihydrochloride, and 5 U HRP. The reaction was initiated by adding 0.5 or 50 mU of purified DAAO contained in potassium phosphate buffer for D-alanine or D-glutamate kinetics, respectively. For substrate specificity profiling of DAAO, 10 mM D-amino acid and 5 mU DAAO were used, with all other assay components as described above.

For measurement of the kinetic parameters of DAAT or its mutants, reaction mixtures consisted of 16 μM pyridoxal 5′-phosphate monohydrate (PLP), 1 μM FAD, 0.5 mM *o*-dianisidine dihydrochloride, and 5 U HRP. Additionally, the reaction mixtures contained varying concentrations of the donor or acceptor substrate being studied and either 15 or 10 mM of the donor (D-glutamate) or acceptor substrate (pyruvate), respectively. Reactions were initiated by the addition of 2 mU of purified DAAT and 200 mU of purified DAAO contained in 70 μL potassium phosphate buffer.

For all kinetic experiments described above, separate reactions in which the enzyme being studied was not included were used as blanks. All reactions were monitored by measuring the absorbance of the bisazobiphenyl species [15] produced by the oxidation of *o*-dianisidine by HRP at 450 nm (ε = 11.3 mM^{-1} cm^{-1} [16]) every 12 seconds for 30 minutes in individual wells of 96-well plates (Greiner Bio-One) using an Infinite M1000 plate reader

(Tecan). Path lengths for each well were calculated ratiometrically using the difference in absorbance of the reaction mixture at 998 nm and 900 nm. Apparent initial rates were measured from the slope of the linear phase of the reaction over 25-35 data points (300-420 seconds). Nonlinear regression analysis of the apparent initial rates as a function of substrate concentration was performed using the GraphPad Prism software.

2.2.6 Preparation of clarified cell lysates

The DNA libraries described above were transformed into *E. coli* BL21-Gold(DE3) cells (Stratagene) and colonies were picked into individual wells of V96 MicroWell polypropylene plates (Nunc) containing 250 µL of LB supplemented with 100 µg/mL ampicillin. The plates were covered with a sterile breathable rayon membrane (VWR) and incubated overnight at 37 °C with shaking. A mother plate was created by adding 100 µL of this cell culture to 100 µL of a 50% glycerol, 1% NaCl solution in a separate microplate. This mother plate was used to inoculate Nunc V96 MicroWell plates containing 300 µL of Overnight Express Instant TB Medium (Novagen) supplemented with 100 µg/mL ampicillin per well. The resulting "daughter" plates were then covered with a breathable rayon membrane and incubated overnight at 37 °C with shaking. After incubation, cells were harvested by centrifugation (3000 × g, 30 minutes, 4 °C), and the pellets were washed twice with phosphate-buffered saline, pH 7.4. Washed cell pellets were resuspended in lysis buffer (100 mM potassium phosphate buffer pH 8, 1X Bug Buster Protein Extraction Reagent (Novagen), 25 U/mL Benzonase Nuclease (EMD), and 1 mg/mL lysozyme). The clarified lysate was collected by centrifugation as described above and stored at 4 °C until screening.

2.2.7 Screening assays

All assays were performed in triplicate 200-μL reactions at 37 °C in 100 mM potassium phosphate buffer (pH 8.0) by using lysates prepared from three separate "daughter" plates. For screening with the DAAO coupled assay, reaction mixtures contained 15 mM D-glutamate, 10 mM pyruvate or phenylpyruvate, 16 μM PLP, 1 μM FAD, 0.5 mM *o*-dianisidine dihydrochloride, and 5 U HRP. 96-well plates containing the reaction mixture were incubated at 37 °C for 10 minutes, after which the reaction was initiated by the addition of 25 μL of clarified cell lysates prepared as described above. The reactions were monitored for 30 minutes as described for steady-state kinetics. Screening of the DAAT-Y31X saturation library with the GDH assay was performed as previously described [9].

2.3 Results & Discussion

2.3.1 Coupled assay optimization

Our goal was to develop an assay for the screening of DAAT mutant libraries that would be more sensitive than the GDH assay that we developed previously [9] but that would maintain all of its advantages including its high-throughput, ease of implementation, continuous detection of product, and ability to screen many desired substrate candidates. To meet these requirements, we elected to couple the DAAT activity with that of *Trigonopsis variabilis* DAAO, an enzyme that oxidizes efficiently a broad range of D-amino acids into their corresponding α-keto acids (Figure 2.1 and [17; 18; 19]), enabling the detection of a variety of DAAT products. Additionally, this coupling enzyme was selected because it reacts very poorly with D-glutamate, the donor substrate of DAAT, having a k_{cat}/K_M value for this substrate that is over 200-fold lower than that of its native substrate D-alanine (Table 2.1). In

the DAAO coupled assay (Figure 2.2), DAAT transfers the amino group of D-glutamate to pyruvate to generate α-ketoglutarate and D-alanine. D-alanine is then oxidized back into pyruvate by DAAO in the presence of molecular oxygen and water with concomitant release of ammonium and hydrogen peroxide, which can be detected colorimetrically by the addition of HRP and *o*-dianisidine.

Table 2.1 Apparent kinetic parameters of *Trigonopsis variabilis* DAAO for the oxidation of D-amino acids

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
D-Alanine	16 ± 2	49 ± 2	3063
D-Glutamate	62 ± 9	0.85 ± 0.08	14

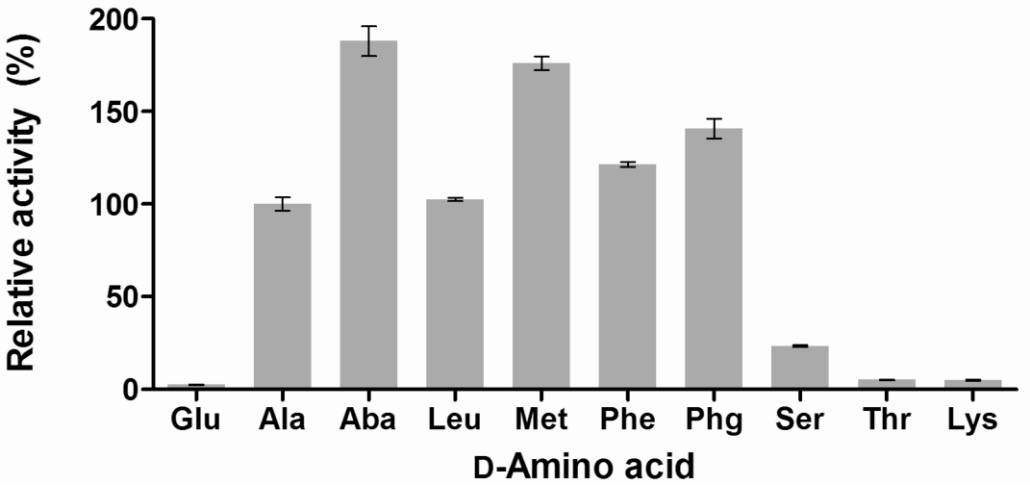

Figure 2.1 Substrate specificity of *Trigonopsis variabilis* DAAO. Activity values for various D-amino acids relative to that of native substrate D-alanine are reported. D-2-aminobutyric acid and D-phenylglycine are identified as Aba and Phg, respectively. All activity assays were performed in triplicate as described under Methods.

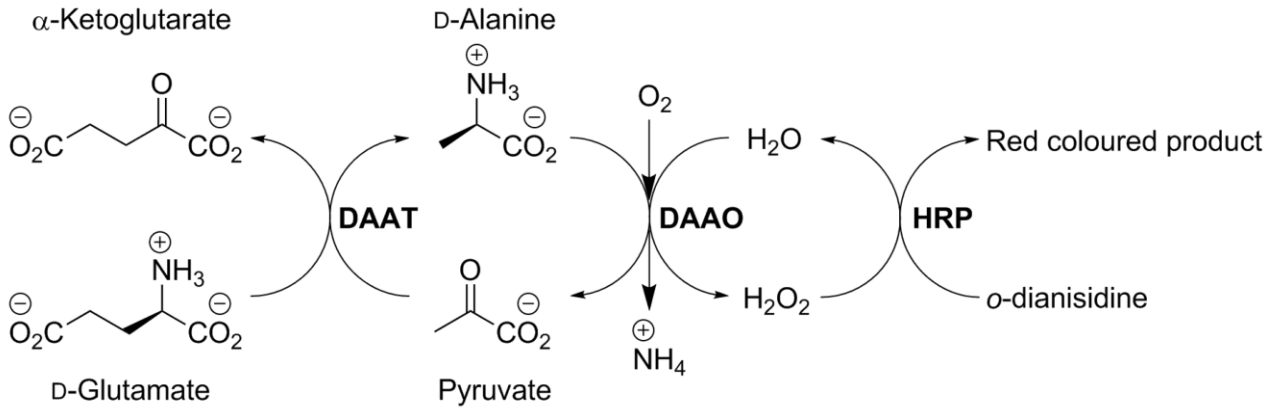

Figure 2.2 DAAO coupled enzyme assay. The transamination reaction catalyzed by DAAT produces D-alanine, which is oxidized back to pyruvate by DAAO in the presence of molecular oxygen and water. The DAAO reaction produces hydrogen peroxide, which is used by HRP to oxidize *o*-dianisidine into a red coloured product.

As a first step in the development of the DAAO screening assay, reaction conditions were optimized using purified *Bacillus* sp. YM-1 DAAT [20], *Trigonopsis variabilis* DAAO [17], and HRP to ensure that the DAAT reaction was the rate-limiting step. To do this, we initially assayed 2 mU of DAAT with large excesses of the coupling enzymes (≥ 100-fold) and saturating amounts of the FAD cofactor of DAAO (K_D = 0.15 ± 0.01 µM [21]) and the *o*-dianisidine substrate of HRP (K_M = 17.3 ± 0.6 µM [22]). Additionally, we used 16 µM of the PLP cofactor of DAAT since this concentration is sufficient to enable maximum transamination activity [9]. As can be seen in Figure 2.3, no significant changes in rate were observed over a wide range of FAD, HRP, and *o*-dianisidine concentrations. However, a higher rate was observed when the DAAO concentration was increased to 400 mU (Figure 2.3A), a concentration 200-fold higher than that of DAAT. This observed rate increase

results from higher competing reactivity of DAAO with D-glutamate, which can become significant at very high coupling enzyme concentrations. Nevertheless, with a 100-fold excess of DAAO relative to DAAT, the rate increases linearly with increased DAAT concentration (Figure 2.3E), demonstrating that assay conditions (2 mU DAAT, 16 μM PLP, 200 mU DAAO, 1 μM FAD, 0.5 mM *o*-dianisidine, and 5 U HRP) are optimal for ensuring that the DAAT reaction is the rate-limiting step. This observation is further supported by the fact that Michaelis-Menten plots (Figure 2.4) show the expected hyperbolic relationship between initial rates and aminotransferase substrate concentrations, allowing for apparent kinetic parameters of the DAAT-catalyzed transamination of pyruvate with D-glutamate to be determined (Table 2.2).

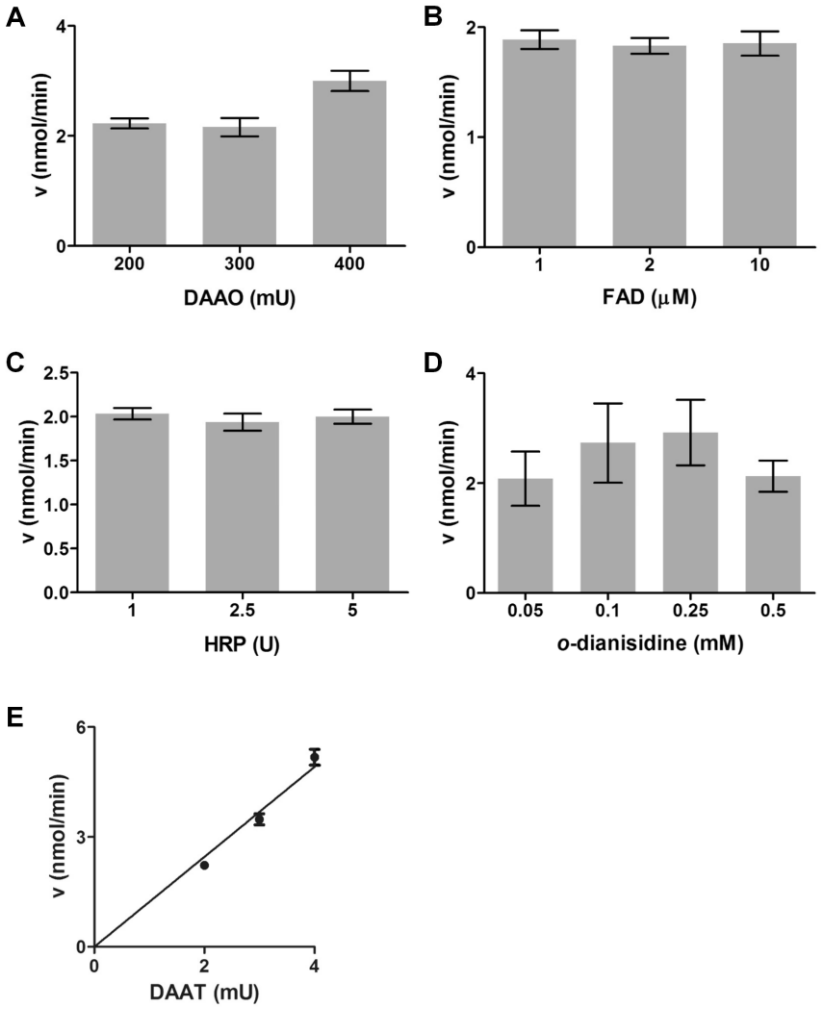

Figure 2.3 Optimization of the DAAO coupled assay. Shown are the rates obtained when the concentrations of DAAO (A), FAD (B), HRP (C), *o*-dianisidine (D), and DAAT (E) are varied while maintaining that of the other components constant at the following values: 2 mU DAAT, 16 μM PLP, 15 mM D-glutamate, 10 mM pyruvate, 200 mU DAAO, 1 μM FAD, 0.5 mM *o*-dianisidine, and 5 U HRP. One unit (U) is defined as the amount of enzyme that catalyzes the conversion of 1 μmole of substrate into product per minute. For the rates measured with varying amounts of DAAT (E), a 100-fold higher concentration of DAAO (200-400 mU) was used. All experiments were performed in triplicate.

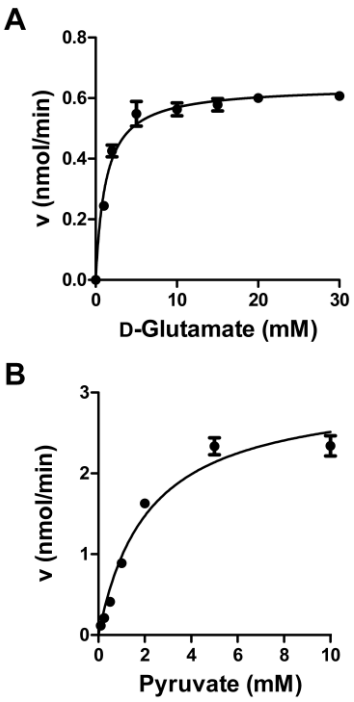

Figure 2.4 Steady-state kinetics of *Bacillus* sp. YM-1 DAAT measured using the DAAO coupled enzyme assay. Michaelis-Menten plots of initial rates as a function of D-glutamate (A) or pyruvate (B) concentrations. All experiments were performed in triplicate.

Table 2.2 Apparent kinetic parameters of *Bacillus* sp. YM-1 DAAT and its mutants for the transamination of pyruvate with D-glutamate.

Enzyme	D-Glutamate				Pyruvate			
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	(k_{cat}/K_M wild type)/ (k_{cat}/K_M mutant)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	(k_{cat}/K_M wild type)/ (k_{cat}/K_M mutant)
Wild type	1.4 ± 0.4	0.5 ± 0.1	357	-	2.08 ± 0.08	0.58 ± 0.01	279	-
Y31P	1.08 ± 0.08	0.00056 ± 0.00001	0.52	687	0.521 ± 0.002	0.00051 ± 0.00002	0.98	285
Y31R	3.9 ± 0.4	0.0007 ± 0.0001	0.18	1983	1.82 ± 0.06	0.00064 ± 0.00002	0.35	797

2.3.2 High-throughput screening

Next, we used the optimized DAAO assay conditions to develop a high-throughput screening procedure that would enable the detection of DAAT activity in cell lysates. To increase throughput, enzyme samples should not need to be purified in order for their catalytic activity to be adequately detected. Thus, clarified *E. coli* cell lysates containing overexpressed DAAT were used as samples. Furthermore, to decrease costs, the DAAO coupling enzyme was co-expressed with DAAT in *E. coli* cells using the dual expression vector pETDuet-1, which is designed for the co-expression of two desired proteins. This vector contains two multiple cloning sites preceded by a T7 promoter and a *lac* operator, which can be used to induce the expression of two separate proteins with IPTG. An additional benefit of co-expressing DAAO with DAAT is that it eliminates the need for the expensive FAD cofactor to be added to the screening reaction mixtures as it is endogenously produced by *E. coli* at a concentration sufficient for ensuring DAAO activity [23]. As a negative control we also prepared a pETDuet-1 vector containing only the DAAO gene and not the DAAT gene since DAAO can react, albeit slightly, with D-glutamate (Figure 2.1 and Table 2.1).

Using clarified cell lysates prepared from *E. coli* cells overexpressing both wild-type DAAT and DAAO, as well as from the negative control described earlier, we performed the DAAO assay as described under Methods. An additional negative control that consisted of a clarified cell lysate from *E. coli* cells harbouring a pETDuet-1 vector that did not contain either the DAAT or DAAO genes was also tested. For these assays, we used 15 mM D-glutamate and 10 mM pyruvate, concentrations representing ≈10 and ≈5 × K_M for the donor and acceptor substrates, respectively. The results, shown as black lines on Figure 2.5A, show

a large absorbance increase with a lysate that contains both wild-type DAAT and DAAO and no signal in the absence of both DAAT and DAAO, confirming that neither of these enzymes are present in the *E. coli* genome [24]. A significant increase in absorbance over time is also observed when DAAO is overexpressed alone, indicating that DAAO does react with the D-glutamate present in the reaction mixture. However, the activity of the cell lysate containing both DAAT and DAAO (2.2 ± 0.3 mU min^{-1}) is ≈8-fold higher than that of the lysate that contains only DAAO (0.28 ± 0.01 mU min^{-1}), which is expected since DAAT reacts much more efficiently with D-glutamate than DAAO. Thus, although DAAO does react with D-glutamate under these conditions, it does not prevent detection of DAAT activity from clarified cell lysates containing small quantities of this enzyme.

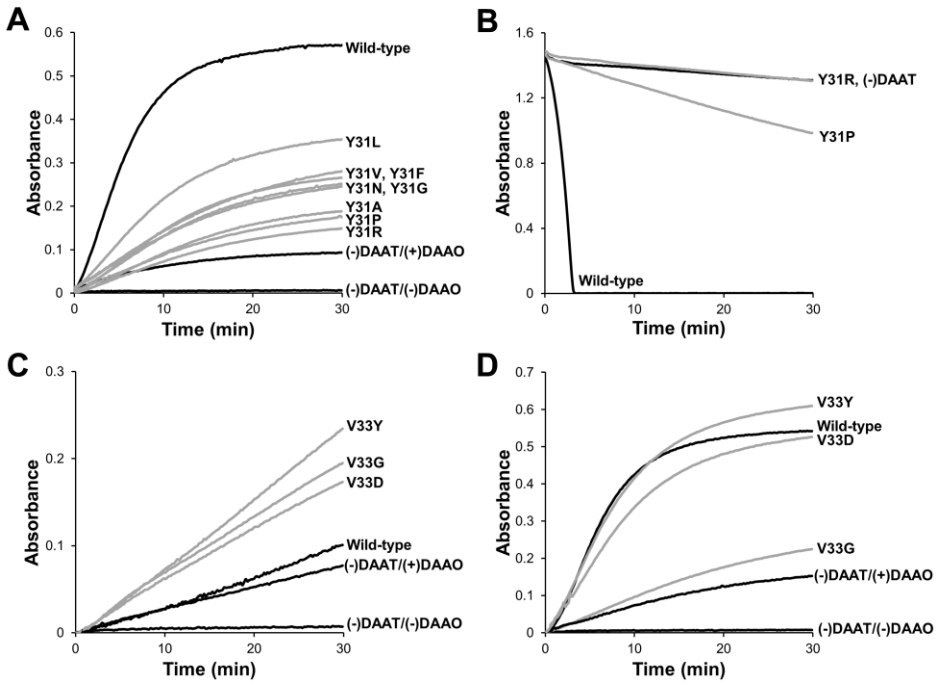

Figure 2.5 Screening of DAAT mutant libraries. The DAAO or GDH coupled assays were used to screen clarified *E. coli* lysates expressing the *Bacillus* sp. YM-1 DAAT saturation libraries. (A) Screening of the DAAT-Y31X saturation library with the DAAO coupled assay for the transamination of pyruvate with D-glutamate. (B) Screening of the DAAT-Y31X saturation library with the GDH coupled assay [9] for the transamination of pyruvate with D-glutamate. (C) Screening of the DAAT-V33X saturation library with the DAAO coupled assay for the transamination of non-native acceptor phenylpyruvate with D-glutamate. (D) Screening of the DAAT-V33X saturation library with the DAAO coupled assay for the transamination of pyruvate with D-glutamate. In all cases, black lines represent positive and negative controls whereas grey lines represent select mutants discussed in text. Positive controls, which are labelled "Wild-type", consist of clarified cell lysates containing overexpressed wild-type DAAT (B) or both wild-type DAAT and DAAO (A, C, and D). Negative controls consist of lysates from cells that do not overexpress DAAT or both DAAT and DAAO.

To evaluate the sensitivity of the DAAO screening assay, we used it to screen a DAAT mutant library in which the Y31 residue is replaced by all other proteinogenic amino acids. This DAAT-Y31X saturation library was selected because we had previously screened it using the GDH assay [9], allowing for a direct comparison of results. A total of 90 variants from the DAAT-Y31X library were screened using the DAAO assay in a 96-well plate, a number approximately three times that of the theoretical library size which ensured that ≈95% of the expected diversity was tested [25]. In this assay, only variants whose activity was greater than the negative control that contained DAAO but not DAAT were considered active (Figure 2.5A). Following screening, eight active mutants were identified (Y31L, Y31V, Y31F, Y31N, Y31G, Y31A, Y31P, and Y31R), two more than had been previously isolated when this library was screened with the GDH assay [9]. The first of these two newly identified active mutants, Y31N, may not have been identified as an active mutant when the library was screened with the GDH assay because it was not included in those screened, likely being part of the ≈5% of variants expected to be absent from the screening plate. The second active mutant identified with the DAAO assay but not the GDH assay is the lowest activity mutant Y31R. Screening of the DAAT-Y31X saturation library with the GDH assay gave a signal for the Y31R mutant that was indistinguishable from that of the negative control (Figure 2.5B), leading to the classification of this mutant as inactive. This is in contrast with the signal obtained for the second lowest activity mutant Y31P, which could be clearly distinguished from the negative control in both the GDH and DAAO assays (Figure 2.5, A and B). It is important to note that we screened the DAAT-Y31X library with the DAAO assay in triplicate by using lysates extracted from three different daughter plates

prepared from the same mother plate, and that this resulted in the identification of the same eight active mutants, demonstrating the reproducibility of our assay.

The fact that we detected the catalytic activity of the Y31R mutant using the DAAO assay but not the GDH assay suggested to us that the DAAO assay is more sensitive. To determine whether this was true, we expressed and purified the two lowest activity mutants (Figure 2.5A, Y31P and Y31R) and measured their apparent kinetic parameters using the DAAO assay (Table 2.2). For these assays, the donor or acceptor substrate concentration was varied while keeping the concentration of the other substrate fixed at the value used during screening (15 mM for D-glutamate and 10 mM for pyruvate) in order to remain consistent with the screening conditions, thereby allowing a more direct comparison with the activities observed during screening. For Y31P, we measured a k_{cat}/K_M of 0.52 M^{-1} s^{-1} for the D-glutamate donor, a value that is 687-fold lower than that of the wild type. This relative decrease in k_{cat}/K_M is in good agreement with the 632-fold decrease obtained with the GDH assay [9]. For Y31R, we obtained a k_{cat}/K_M of 0.18 M^{-1} s^{-1}, a value that is 1,983-fold lower than that of the wild-type. This result confirms that the DAAO assay is more sensitive than the GDH assay, since it allowed the detection of a mutant with a ≈3-fold lower k_{cat}/K_M than the lowest-activity mutant identified with the GDH assay. The increased sensitivity of the DAAO assay likely results from the fact that this assay produces a signal increase instead of a signal decrease, as is the case with the GDH assay, thereby allowing the detection of lower activity enzymes. An additional factor contributing to the increased sensitivity of the DAAO assay is that its absorbance increase is produced at a wavelength in the visible range (450 nm) where there is little background signal in cell lysates. This is in contrast with the GDH

assay in which absorbance is measured at a wavelength in the near-UV (340 nm) where many cell components are known to absorb.

2.3.3 Substrate specificity screening

Having demonstrated that the DAAO screening assay could be used to detect active DAAT mutants from cell lysates, we investigated whether the assay could also be used for the identification of mutants displaying altered substrate specificity, which is a desirable goal in enzyme engineering. For this application, the native acceptor substrate pyruvate is replaced by a desired alternate α-keto acid, resulting in the synthesis of a different D-amino acid via the transamination activity of mutant DAATs. To demonstrate that the DAAO assay can be used for the detection of altered specificity mutants, we screened a saturation library prepared by mutating residue V33 to all other proteinogenic amino acids. This residue was selected because it is located in the D-amino acid substrate side-chain binding pocket of the DAAT active site [26] and because it has been postulated to hinder access to the side-chain binding pocket for bulky D-amino acid substrates [27]. Thus, we hypothesized that replacement of V33 by a smaller amino acid would increase the size of the side-chain binding pocket, allowing for more efficient binding of larger α-keto acids.

To test this hypothesis, the DAAT-V33X saturation library was screened with the alternate acceptor phenylpyruvate. Phenylpyruvate was selected because it is bulkier than pyruvate and because it is a much lower activity substrate of wild-type DAAT, having an 85-fold lower k_{cat}/K_M than the native substrate [9]. Since the benzyl side chain of phenylpyruvate is larger than the methyl side chain of pyruvate, it is likely that the lower activity of wild-type DAAT for this alternate substrate results from increased steric clashes

between the substrate and the enzyme. Additionally, phenylpyruvate was selected as an alternate acceptor because its transamination product D-phenylalanine is a highly active substrate of DAAO (Figure 2.1), enabling its detection with the DAAO assay.

Following screening of the DAAT-V33X saturation library with phenylpyruvate, we identified three mutants that were significantly more active than the wild type with this bulkier acceptor substrate (Figure 2.5C). These mutants, V33Y, V33G, and V33D, displayed activities of 0.27 ± 0.06, 0.272 ± 0.002, and 0.24 ± 0.03 mU min^{-1}, respectively, representing a ≈1.5-fold activity increase relative to the wild type (0.17 ± 0.03 mU min^{-1}). Since the higher activity observed with phenylpyruvate for these mutants could result from higher quantities of soluble enzyme in the tested lysates, we also screened identical volumes of these lysates with native acceptor pyruvate. As can be seen on Figure 2.5D, the activity levels obtained for each mutant by screening with pyruvate is either similar to (V33Y) or lower than (V33D and V33G) that of the wild type. These results suggest that the increased activities observed for the mutants relative to wild type when screened with phenylpyruvate were not caused by higher enzyme concentrations in the lysates but by higher activity towards this non-native acceptor, which could be indicative of an altered substrate specificity.

To confirm the observed altered substrate specificity, we expressed and purified the two most active mutants found by screening with phenylpyruvate (V33G and V33Y) and measured their apparent kinetic parameters using the DAAO coupled assay (Table 2.3). The V33G and V33Y mutants display k_{cat}/K_M values for phenylpyruvate of 48 M^{-1} s^{-1} and 43 M^{-1} s^{-1}, respectively, which are ≈3-fold higher than that of the wild type. These results confirm that these mutants are indeed more efficient at transaminating phenylpyruvate than the wild

type. Interestingly, the V33G mutant displays a ≈1.3-fold lower k_{cat}/K_M value for native substrate pyruvate (36 M^{-1} s^{-1}) than for phenylpyruvate, indicating that it reacts more efficiently with the bulkier substrate than the smaller one. This result may be explained by the fact that the wild-type valine residue at position 33 is replaced in this mutant by the smallest amino acid, glycine. This mutation likely creates a larger substrate side-chain binding pocket, reducing steric clashes for bulky substrates and decreasing binding affinity for small ones. This supposition is confirmed by the fact that the K_M value of the V33G mutant for the smaller acceptor substrate pyruvate is higher than that of the wild type, while its K_M value for the bulky phenylpyruvate is lower (Table 2.3). Additionally, the V33G mutant displays an ≈8-fold decrease in k_{cat}/K_M for pyruvate relative to wild type, which taken together with the ≈3-fold increase in k_{cat}/K_M for phenylpyruvate, results in a ≈25-fold specificity switch relative to the wild type, making this mutant more specific for phenylpyruvate than native substrate pyruvate, unlike wild-type DAAT which displays the opposite preference. On the other hand, the V33Y mutant maintains high catalytic efficiency for the transamination of pyruvate (k_{cat}/K_M = 352 M^{-1} s^{-1}) yet is also ≈3-fold more efficient than the wild type at transaminating phenylpyruvate, demonstrating a broadened acceptor substrate specificity. It is unclear how the V33Y mutation would increase reactivity with the bulkier acceptor phenylpyruvate since the tyrosine side chain is larger than that of valine. Inspection of the DAAT active site pocket [26] reveals that there is little space to accommodate both bulky aromatic groups, suggesting that a conformational change may be required to enable the V33Y mutant to react with phenylpyruvate. Nevertheless, kinetic characterization of the V33G and V33Y mutants confirmed that the DAAO assay developed herein can be used to identify DAAT mutants displaying altered substrate specificities.

Table 2.3 Apparent kinetic parameters of *Bacillus* sp. YM-1 DAAT and its mutants for the transamination of pyruvate or phenylpyruvate with D-glutamate.

Enzyme	Pyruvate			Phenylpyruvate			
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	(k_{cat}/K_M pyruvate) / (k_{cat}/K_M phenylpyruvate)
Wild type[a]	2.08 ± 0.08	0.58 ± 0.01	279	6.7 ± 0.9	0.091 ± 0.008	14	20
V33G[b]	4.7 ± 0.1	0.17 ± 0.01	36	2.1 ± 0.3	0.10 ± 0.07	48	0.8
V33Y[c]	0.27 ± 0.02	0.095 ± 0.004	352	1.5 ± 0.4	0.064 ± 0.009	43	8

[a] This enzyme displays substrate inhibition for phenylpyruvate with a K_i of 1.1 ± 0.1 mM.
[b] This enzyme displays substrate inhibition for phenylpyruvate with a K_i of 1.4 ± 0.4 mM.
[c] This enzyme displays substrate inhibition for phenylpyruvate with a K_i of 2.9 ± 0.6 mM

Previously, Gutierrez *et al.* engineered a DAAT variant displaying broadened substrate specificity by replacing the core motif of the interdomain loop by three glycine residues, resulting in the P119G/R120G/P121G triple mutant [28]. This triple mutant displays a 2.8-fold increase in specific activity towards D-phenylalanine relative to the wild type, a result similar to the phenylpyruvate k_{cat}/K_M increase observed for the V33G and V33Y mutants developed herein. Since the interdomain loop residues are not in direct contact with the substrate or cofactor, in contrast to residue V33 which is located directly next to the substrate, it is likely that the broadened specificities of the P119G/R120G/P121G and V33G/Y mutants are achieved by different mechanisms. Thus, it may be possible to further increase the activity of DAAT towards non-native substrates by combining these different sets of mutations in an additional round of rational design, or by using these mutants as starting points for directed evolution.

2.3.4 Advantages of the DAAO coupled assay for mutant library screening

As a screening assay for DAAT mutant libraries, the DAAO coupled assay developed herein has many benefits. It is a high-throughput method, as 96 samples can be tested at a time, and $>10^3$ mutants can be screened in a matter of hours. Our assay is continuous, allowing the kinetics of mutant DAATs to be evaluated semi-quantitatively during screening, providing information on their relative catalytic efficiency. This assay also has the benefit of allowing the evaluation of many candidate substrates of DAAT due to the broad substrate specificity of DAAO, thereby enabling the identification of mutants displaying altered substrate specificity. While these advantages are also provided by the GDH assay, the DAAO assay developed here is more sensitive due to its capacity to generate a signal

increase instead of decrease as is the case for the GDH assay. Sensitivity of the DAAO assay may be further increased by replacing *o*-dianisidine by Amplex® Red [29], an alternate substrate of HRP that is oxidized into the highly fluorescent molecule resorufin. Doing so would enable fluorescence detection, which typically results in an order of magnitude sensitivity increase relative to absorption [30]. A further benefit of the DAAO assay is that it allows for the detection of the D-amino acid product of DAAT instead of α-ketoglutarate, as is the case with the GDH assay. Detection of the D-amino acid product confirms its stereochemical configuration, which could be used to evaluate the enantioselectivity of other aminotransferases, for example when studying the stereoinverting D-phenylglycine aminotransferase, an enzyme that produces D-phenylglycine via transamination of benzoylformate with L-glutamate [31].

2.4 Conclusion

We have developed a continuous, high-throughput assay for the screening of DAAT mutant libraries that is based on the use of the broad specificity DAAO from the yeast *Trigonopsis variabilis*. This assay is of broad utility as it may be used for the screening of many α-keto acid acceptor substrates. It is also sensitive as it allowed us to readily detect a mutant with ≈2,000-fold lower catalytic efficiency than the wild type in a complex reaction mixture containing small quantities of enzyme. Finally, using this assay, we were able to identify two DAAT mutants (V33G and V33Y) that are more efficient at catalyzing the transamination of phenylpyruvate with D-glutamate than the wild type. We expect that our high-throughput assay will be useful for the engineering of additional DAAT mutants displaying increased catalytic activity towards non-native substrates.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada, the University of Ottawa, and the Canada Foundation for Innovation.

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3. Substrate specificity profiling of D-amino acid aminotransferase mutants displaying increased activity for the non-native substrate phenylpyruvate

Preface

Following the development of the DAAO assay and the identification of three DAAT mutants displaying increased activity towards the non-native substrate phenylpyruvate, it became of interest to further characterize these mutants for their applicability as biocatalysts for the production of enantiopure D-amino acids. However, we were limited by the fact that the DAAO assay allows only for the screening of DAAT activity with various α-keto acids, which can be expensive to purchase, or not available commercially. Therefore, we developed another assay, using DDO as a coupling enzyme. As DDO oxidizes only D-glutamate and D-aspartate, we were able to test the specificity of DAAT with a wide range of D-amino acids to further characterize the substrate specificity profile of the wild type enzyme and the V33D, V33G, and V33Y mutants. This chapter is written as a manuscript for future journal submission.

3.1 Introduction

Enantiopure amino acids are essential building blocks for the development and synthesis of antibiotics and peptidomimetic drugs. The use of D- [1] and β- [2] amino acids in peptide-based drugs is especially useful, as the resulting biologics are resistant to proteolytic

degradation. Biocatalysis represents a promising method to provide the industry with enantiopure amino acids, due to the high stereospecificity of enzymes which enables the formation of an optically pure product that can be difficult to obtain through traditional organic synthesis. D-Amino acids are especially in demand, and while biocatalytic processes exist to prepare some of these compounds [3, 4], high yields are not possible for all D-amino acids. Therefore, there is still interest in developing biocatalysts for the production of these valuable compounds.

Biocatalytic processes that incorporate the use of D-amino acid aminotransferase (DAAT) from *Bacillus* species for the production of enantiopure D-amino acids have been previously described [5, 6, 7]. The crystal structure of DAAT from *Bacillus sp.* YM-1 is known [8], and the broad substrate specificity of this enzyme has made it an attractive candidate for use as a biocatalyst. However, while DAAT will catalyze the formation of D-phenylalanine from phenylpyruvate, it does so with a catalytic efficiency that is approximately 85-fold lower than for the formation of D-glutamate from α-ketoglutarate [9]. The lower activity of DAAT towards aromatic amino acids is further illustrated by the fact that a 10-fold higher amount of DAAT was required for the synthesis of D-phenylalanine than for the synthesis of D-glutamate using a biocatalytic system employing this enzyme [6]. To address this issue, it would be useful to engineer DAAT variants with increased activity towards D-phenylalanine and other aromatic D-amino acids.

Previously, we identified three mutants of DAAT (V33D, V33G, and V33Y) that display increased activity towards an aromatic substrate [10]. Kinetic characterization of two of these mutants, V33G and V33Y, showed that both mutants were ≈3-fold more active towards the non-native acceptor phenylpyruvate than wild type. Therefore, it became of

interest to further characterize the kinetic properties and substrate specificity of these mutants to evaluate their potential as biocatalysts for the synthesis of aromatic D-amino acids.

In this study, we present the development of a new coupled assay to detect DAAT activity towards a wide range of D-amino acids and its application in the substrate specificity profiling of the DAAT V33D, V33G, and V33Y mutants. The coupled assay described herein is based on the use of D-aspartate oxidase (DDO), an enzyme that oxidizes specifically D-aspartate and D-glutamate to their corresponding α-keto acids. Unlike the DAAO assay described in Chapter 2, the DDO assay allows for the detection of DAAT activity with a wide range of D-amino acid donor substrates, as opposed to different α-keto acid acceptors. Using this assay, we were able to determine the specific activity of wild-type DAAT and its mutants for several D-amino acids, including unnatural D-phenylalanine derivatives.

3.2 Materials and Methods

3.2.1 Materials

All reagents used were of the highest available purity. Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs. Synthetic oligonucleotides were obtained from Integrated DNA Technologies, and Ni-NTA agarose resin was obtained from Promega. All aqueous solutions were prepared using water purified with a Barnstead Nanopure Diamond system. Amino and keto acids, cofactors, *o*-dianisidine, horseradish peroxidase (HRP), and lysozyme were purchased from Sigma-Aldrich with the exception of

O-methyl-D-tyrosine (Santa-Cruz Biotechnologies), 3-methyl-D-phenylalanine, and 4-methyl-D-phenylalanine (Alfa Aesar).

3.2.2 Preparation of plasmid constructs

A codon-optimized gene for His-tagged *Bos taurus* DDO obtained from GenScript was sub-cloned into pET11-a (Novagen) via *Nde*I/*Bam*HI restrictions sites. This plasmid was transformed into *E. coli* XL1-Blue cells (Stratagene) and the entire open reading frame was verified by sequencing.

3.2.3 Protein expression and purification

Proteins were expressed in 500 mL or 1 L cultures (LB with 100 μg/mL ampicillin) of *E. coli* BL21-Gold(DE3) cells (Stratagene) transformed with either the pETDuet-DAAO-DAAT vector containing a mutant DAAT gene, or pET11a containing the wild-type DAAT or DDO genes. Cultures were grown at 37 °C with shaking until an optical density of 0.6 at 600 nm was reached, at which point protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells expressing DAAT or one of its mutants were incubated for 3 hours at 37 °C with shaking, while cells expressing DDO were incubated overnight at 17 °C with shaking. Following incubation, cells were harvested by centrifugation and lysed with an EmulsiFlex-B15 cell disruptor (Avestin). Proteins were purified by immobilized metal affinity chromatography according to the manufacturer's protocol. The eluted fractions were desalted by gel filtration using EconoPAC 10DG columns (Bio-Rad) into 100 mM potassium phosphate buffer (pH 8.0). Protein concentrations were quantified using a

modified Bradford assay, where the calibration curve is constructed as a plot of the ratio of absorbance at 590 nm and 450 nm versus concentration [11].

3.2.4 Steady-state kinetics

One unit (U) is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate into product per minute. All kinetic assays were performed in triplicate 200-μL reactions at 37 °C in 100 mM potassium phosphate buffer (pH 8.0) and using enzyme from two independent preparations, unless otherwise stated. For the determination of DDO kinetic parameters, reaction mixtures consisted of appropriate amounts of the D-amino acid being studied (D-glutamate or D-aspartate), 1 μM flavin adenine dinucleotide disodium salt hydrate (FAD), 0.5 mM *o*-dianisidine dihydrochloride, and 5 U HRP. For the determination of DDO substrate specificity (Figure 3.1), 10 mM of D-amino acid was used. In all cases, the reaction was initiated by addition of 2 mU purified DDO contained in potassium phosphate buffer.

For measurement of the kinetic parameters of DAAT, reaction mixtures consisted of 16 μM pyridoxal 5′-phosphate monohydrate (PLP), 1 μM FAD, 0.5 mM *o*-dianisidine dihydrochloride, and 5 U HRP. Additionally, the reaction mixtures contained varying concentrations of the donor or acceptor substrate being studied and either 15 or 5 mM of the donor (D-alanine) or acceptor substrate (α-ketoglutarate or oxaloacetate), respectively. Reactions were initiated by the addition of 2 mU of purified DAAT and 25 mU of purified DDO contained in 70 μL potassium phosphate buffer. Kinetics of oxaloacetate were performed using enzyme from only one preparation.

For all kinetic experiments described above, separate reactions in which the enzyme being studied was not included were used as blanks. All reactions were monitored by

measuring the absorbance of the bisazobiphenyl species [12] produced by the oxidation of *o*-dianisidine by HRP at 450 nm (ε = 11.3 mM^{-1} cm^{-1}[13]) every 12 seconds for 30 minutes in individual wells of 96-well plates (Greiner Bio-One) using an Infinite M1000 plate reader (Tecan). Path lengths for each well were calculated ratiometrically using the difference in absorbance of the reaction mixture at 998 nm and 900 nm. Apparent initial rates were measured from the slope of the linear phase of the reaction over 25-35 data points (300-420 seconds). Nonlinear regression analysis of the apparent initial rates as a function of substrate concentration was performed using the GraphPad Prism software.

3.2.5 DDO pH profile

To determine the optimal pH of DDO, Britton-Robinson buffer was used over a pH range of 2-12. The buffer consisted of a mixture of 40 mM H_3BO_3, 40 mM H_3PO_4, and 40 mM CH_3CO_2H. pH was adjusted to the desired value using 0.2 M NaOH [14]. Activity was assayed using 10 mM D-glutamate as described above.

3.2.6 DAAT Substrate Specificity Profiling

All experiments were performed in triplicate 200 μL reactions at 37°C in 100 mM potassium phosphate buffer (pH 8.0). The reaction mixture consisted of 10 mM D-amino acid, 5 mM oxaloacetate, 16 μM PLP, 1 μM FAD, 0.5 mM *o*-dianisidine dihydrochloride, and 5 U HRP. Reactions were initiated by adding 2 mU of purified wild-type or mutant DAAT and 25 mU of purified DDO contained in 100 μL potassium phosphate buffer. Reactions were monitored and the rates were calculated as described above.

3.3 Results and Discussion

3.3.1 The DDO coupled assay

We previously described a coupled assay for DAAT activity that used *Trigonopsis variabilis* D-amino acid oxidase (DAAO) as a coupling enzyme to detect the formation of the D-amino acid product of the DAAT transamination reaction [10]. This assay proved to be sensitive and useful, allowing us to identify three DAAT mutants (V33D, V33G, and V33Y) that showed higher activity than wild type toward the non-native substrate phenylpyruvate. However, a limitation of this assay is that it can only be used to measure the kinetic parameters of DAAT α-keto acid acceptor substrates due to the required use of the D-glutamate donor (Figure 2.2). This is problematic as most α-keto acids of interest are not commercially available. Therefore, in order to characterize the substrate range of our DAAT mutants, a different assay that enabled the testing of various D-amino acid donors was needed.

To accomplish this goal, we selected D-aspartate oxidase (DDO) from *Bos taurus* [15] as a coupling enzyme. DDO oxidizes specifically D-aspartate and D-glutamate and not other D-amino acids (Figure 3.1). In the DDO coupled assay, DAAT transfers the amino group of a D-amino acid to oxaloacetate, producing D-aspartate and an α-keto acid. D-Aspartate is oxidized back to oxaloacetate by DDO in the presence of molecular oxygen and water with concomitant release of ammonium and hydrogen peroxide, which can be detected colorimetrically by the addition of HRP and *o*-dianisidine (Figure 3.2). Thus, the DDO coupled assay should enable the testing of any desired D-amino acid as a DAAT donor substrate.

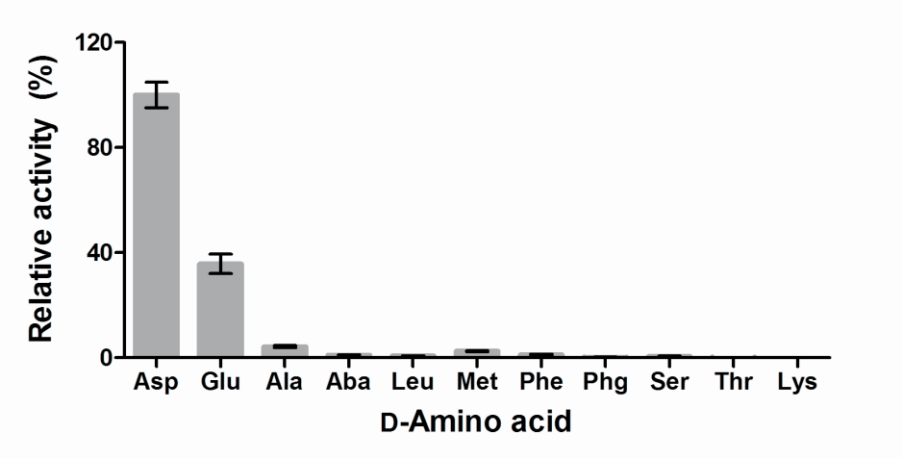

Figure 3.1 Substrate Specificity of *Bos taurus* DDO. Activity values for various D-amino acids relative to that of native substrate D-aspartate are reported. D-2-aminobutyric acid and D-phenylglycine are identified as Aba and Phg, respectively. All activity assays were performed in triplicate.

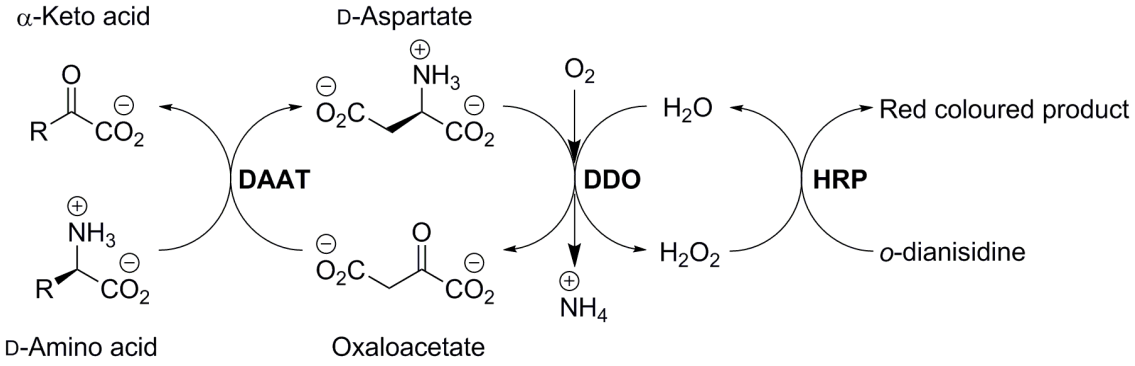

Figure 3.2 DDO coupled enzyme assay. The transamination reaction catalyzed by DAAT produces D-aspartate, which is oxidized back to oxaloacetate by DAAO in the presence of molecular oxygen and water. The DDO reaction produces hydrogen peroxide, which is used by HRP to oxidize *o*-dianisidine into a red coloured product.

3.3.2 Purification and characterization of DDO

In order to incorporate DDO into a coupled assay for DAAT, it was first necessary to express, purify, and characterize this enzyme. Initially, we expressed DDO under identical conditions as those used for DAAT (37ºC, 3 hours). This protocol however resulted in a low yield of 1.4 ± 0.2 mg/L upon purification (Table 3.1), and a low specific activity of 0.33 ± 0.02 U/mg with D-glutamate. Under these conditions, we would not be able to purify enough DDO to use in the large excess required by coupled assays. We speculated that the low yield might be due to the insolubility of the enzyme when overexpressed in *E. coli,* since we observed a much larger band on SDS-PAGE for DDO in the insoluble fraction than in the supernatant (data not shown). Therefore, we modified the expression conditions, allowing DDO to be expressed overnight at 17°C. This change in expression conditions resulted in a 3.6-fold increase in DDO yield (Table 3.1); however, the specific activity remained unchanged (0.39 ± 0.03 U/mg). Although improvements could be achieved by modifying expression conditions further, expression at 17°C allowed us to obtain sufficient amounts of DDO to perform all kinetic assays required to obtain full Michaelis-Menten plots for two substrates. Thus, no additional changes to expression conditions were attempted.

Table 3.1 Optimization of expression conditions for DDO.

Expression Conditions	**Yield (mg/L)**	**Specific Activity (U/mg)**
37°C, 3 h	1.4 ± 0.2	0.33 ± 0.02
17°C, overnight	5.0 ± 0.9	0.39 ± 0.03

As a first step in characterizing recombinant DDO, we assayed its activity with D-glutamate over a pH range of 2-12. As can be seen in Figure 3.3, DDO reaches its maximum

activity at pH 8.0. This optimal pH value is identical to that of DAAT, ensuring that DDO can be used as a coupling enzyme for this aminotransferase. Interestingly, the activity of DDO does not decrease significantly in the pH 8-12 range, indicating that a basic environment is preferred by the enzyme for catalytic activity.

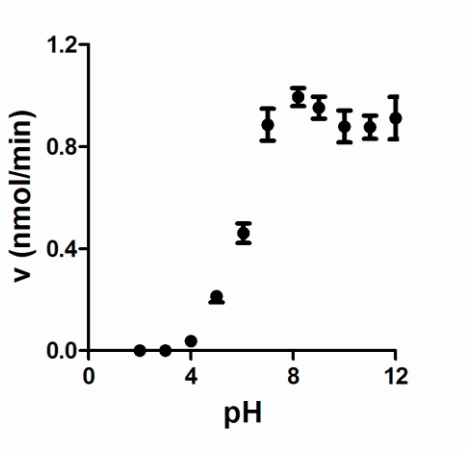

Figure 3.3 pH profile of DDO. Rates obtained when DDO oxidizes D-glutamate over a pH range of 2-12 in Britton-Robinson buffer. All data points represent triplicates.

Next, we determined the kinetic parameters of DDO for D-glutamate and D-aspartate. Although DDO is active with both substrates, it has a k_{cat}/K_M for D-aspartate that is approximately 8-fold higher than for D-glutamate (Table 3.2). The affinity of DDO for D-aspartate and D-glutamate is very similar, as shown by the apparent K_M values of 0.52 ± 0.05 mM and 0.7 ± 0.1 mM, respectively. Therefore, the increase in specificity for D-aspartate relative to D-glutamate is due to the higher turnover number of DDO with D-aspartate. Based on these results, we elected to use oxaloacetate, the α-keto acid of D-aspartate, rather than α-ketoglutarate as the DAAT acceptor in our development of the DDO coupled assay.

Table 3.2 Apparent kinetic parameters of *Bos taurus* DDO for the oxidation of D-amino acids.

Substrate	K_M **(mM)**	k_{cat} **(s^{-1})**	k_{cat}/K_M **($M^{-1}s^{-1}$)**
D-Glutamate	0.7 ± 0.1	0.093 ± 0.002	133
D-Aspartate	0.52 ± 0.05	0.54 ± 0.01	1038

3.3.3 Optimization of the DDO coupled assay

We initially assayed 2 mU DAAT using 15 mM D-alanine and 5 mM oxaloacetate, with all other reaction components in concentrations found to be optimal in the DAAO coupled assay [10] (16 μM PLP, 1 μM FAD, 0.5 mM *o*-dianisidine, 5 U HRP). However due to the lower specific activity and yield of DDO, we could not use a 100-fold excess of this coupling enzyme, and were instead limited to using only a 12.5-fold excess. Although this would result in an underestimation of initial rates, we performed all assays under these conditions, allowing a direct comparison of results. To confirm that the DAAT reaction was the rate-limiting step under these conditions, we doubled the amount of FAD, *o*-dianisidine, or HRP and measured the resulting rate. As shown in Figure 3.4A, no significant change in rate was observed, confirming that the rate measured was that of DAAT. This observation is further supported by the fact that the reaction rate increased linearly with the amount of DAAT used (Figure 3.4B).

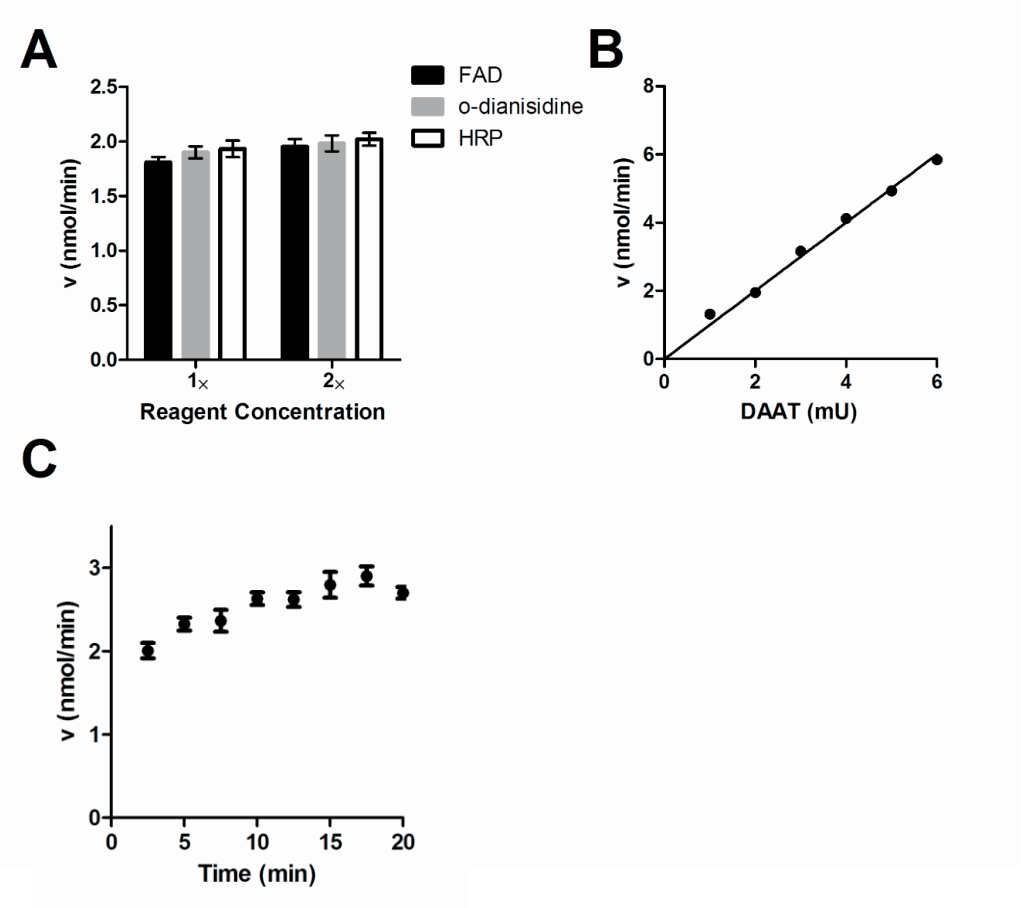

Figure 3.4 Optimization of the DDO coupled assay. (A) Rates obtained for DAAT when the following reagent concentrations are doubled: FAD, 1 μM; *o*-dianisidine, 0.5 mM; HRP, 5U. (B) Rates obtained when increasing amounts of DAAT are assayed under optimal conditions. (C) Rates obtained by initiating the DAAT reaction at different time points following the preparation of a single oxaloacetate stock solution. The reactions were initiated by the addition of 5 mM oxaloacetate to individual wells of a 96-well plate containing identical amounts of all other reagents. One unit (U) is the amount of enzyme that catalyzes the conversion of 1 μmol of substrate into product per minute. All experiments were performed in triplicate.

As mentioned above, oxaloacetate is used as the DAAT acceptor substrate in the DDO assay. However, oxaloacetate spontaneously decarboxylates in solution to release pyruvate and carbon dioxide [16], which would result in underestimation of measured rates. To verify that oxaloacetate decarboxylation would not interfere with our coupled assay, we prepared a single stock solution of this keto acid in buffer and used it to initiate the DAAT reaction at different time intervals following its solubilisation (every 2.5 minutes for 20 minutes). Since all reaction conditions were identical with the exception of the time at which the reaction was initialized, any change in DAAT activity would result from changes in oxaloacetate concentration caused by decarboxylation. As shown in Figure 3.4C, the reaction rate did not decrease over the course of experiment, indicating that decarboxylation of oxaloacetate does not cause an underestimation of DAAT rate. The rate instead appears to increase, which may be due to the longer incubation at 37°C of reaction mixtures containing all components except oxaloacetate found in individual wells of the 96-well plate that was being assayed. Nevertheless, to ensure that oxaloacetate decarboxylation would not affect measured rates, the stock solution of oxaloacetate to be used was always prepared immediately prior to running an assay.

3.3.4 Kinetics of DAAT

Following optimization of the DDO assay conditions, we used the assay to measure apparent kinetic parameters for DAAT (Table 3.3). All Michaelis-Menten plots show the expected hyperbolic shape (Figure 3.5), confirming that the DDO coupled assay is in fact measuring the rate of DAAT. The apparent K_M values of 0.64 ± 0.05 mM and 0.56 ± 0.03 mM (Table 3.3 and Figure 3.5) obtained for D-alanine and α-ketoglutarate, respectively, are similar to

previously reported values of 1.6 mM [17] and 0.63 mM [18]. Conversely, the identical apparent k_{cat} values of 1.18 ± 0.03 mM for both D-alanine and α-ketoglutarate obtained using the DDO coupled assay are two orders of magnitude lower than those previously published. The higher reported apparent k_{cat} values of 280 s^{-1} [17] and 260 s^{-1} [18] are likely due to the fact these were derived from a lactate-dehydrogenase (LDH) coupled assay in which a large (>100-fold) excess of the coupling enzyme was used. When performing the DDO coupled assay however, we were limited to using only a 12.5-fold excess of DDO due to issues with low specific activity as described in section 3.1. Therefore, the lower amount of coupling enzyme that we utilized results in an underestimation of the k_{cat}. Nevertheless, while the apparent k_{cat} values that we obtained for DAAT were much lower than those previously reported in the literature, a direct comparison of results obtained with the DDO coupled assay can still be used to characterize differences in substrate turnover between mutant DAATs.

Table 3.3 Apparent kinetic parameters of *Bacillus* sp. YM-1 DAAT for the transamination of α-keto acids with D-alanine.

Substrate	K_M **(mM)**	k_{cat} **(s^{-1})**	k_{cat}/K_M **($M^{-1}s^{-1}$)**
D-Alanine	0.64 ± 0.05	1.18 ± 0.03	1844
α-Ketoglutarate	0.56 ± 0.05	1.18 ± 0.03	2107
Oxaloacetate	0.97	1.01	1041

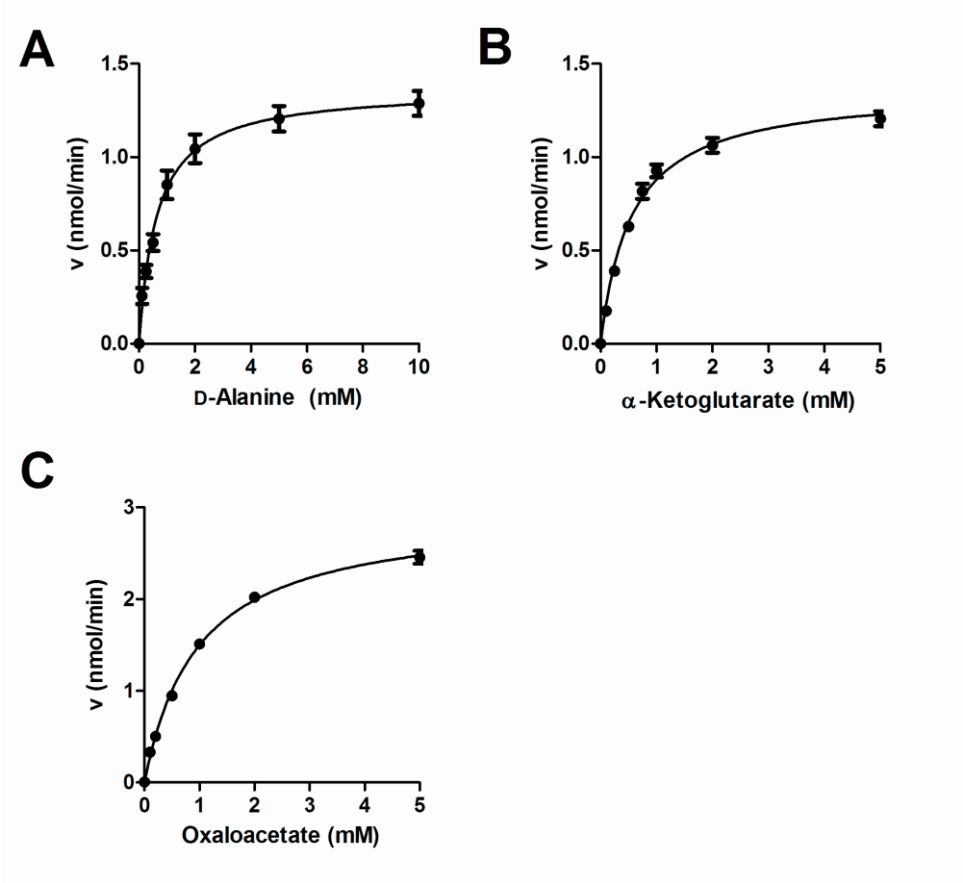

Figure 3.5 Steady-state kinetics of *Bacillus* sp. YM-1 DAAT measured using the DDO coupled enzyme assay. Michaelis-Menten plots of initial rates as a function of D-alanine (A), α-ketoglutarate (B), or oxaloacetate (C) concentrations. All experiments were performed in triplicate.

Finally, we measured apparent kinetic parameters for DAAT with oxaloaceate as an acceptor (Figure 3.5C). An apparent K_M of 0.97 mM was observed, a value which is higher than that of α-ketoglutarate, while the apparent k_{cat} is very similar to that for α-ketoglutarate. No literature data exists for comparison of apparent K_M and k_{cat} values for this substrate. The k_{cat}/K_M for oxaloacetate is approximately 2-fold lower than that for α-ketoglutarate, indicating that DAAT can utilize this substrate efficiently, justifying its use as acceptor in the

DDO assay. Based on this data, we elected to use 5 mM of oxaloacetate when measuring the specific activity of DAAT with various D-amino acid donors, a concentration 5-fold higher than its K_M.

3.3.5 Substrate Specificity of DAAT V33 Mutants

Following validation of the DDO assay, we used it to evaluate the activity of purified wild-type DAAT and its V33D, V33G, and V33Y mutants with a wide range of D-amino acids. Along with many aliphatic and polar D-amino acids, derivatives of D-phenylalanine were also tested to elucidate which types of substitutions on aromatic substrates would be tolerated by our improved mutants. Previously, our group reported the testing of eight D-amino acids as substrates of DAAT using MINISEP-MS, an assay based on capillary electrophoresis coupled to mass spectrometry (CE-MS) [19]. Five of these D-amino acids were identified as being substrates of DAAT (D-alanine, D-valine, D-leucine, D-phenylalanine, D-serine) and three as non-reactive (D-methionine, D-lysine, D-phenylglycine). Our results for wild-type DAAT obtained with the DDO assay (Table 3.4) matched the MINISEP-MS data in seven out of eight cases. The only discrepancy, D-methionine, was identified as a DAAT substrate by our assay but not by MINISEP-MS assay. However, a previously published report identified D-methionine as a substrate of DAAT [17]. Therefore, these results validate the use of the DDO assay as a method for the substrate specificity profiling of DAAT.

As shown on Table 3.4, 16 D-amino acids were tested as substrates of DAAT using the DDO assay. The highest overall specific activity observed was that of wild-type DAAT with its native substrate, D-alanine (2.4 ± 0.5 U/mg). The V33G and V33D mutants displayed 1.9- and 2.4-fold lower specific activities than wild type with respect to D-alanine

whereas V33Y achieved a specific activity comparable to wild type. These results are in good agreement with our previous results obtained using the DAAO assay (Chapter 2) which showed that the V33Y mutant displayed an approximately 1.3-fold higher k_{cat}/K_M than wild type, while V33G displayed an approximately 8-fold lower k_{cat}/K_M than wild type. The lower activity of V33G with the small D-alanine substrate was attributed to a larger substrate side-chain binding pocket that decreases binding affinity for small substrates via a loss of stabilizing interactions [10].

Table 3.4 Specific activity of *Bacillus* sp. YM-1 DAAT and its mutants for the transamination of oxaloacetate with a variety of D-amino acids.

	Specific Activity (U/mg)			
D-Amino acid	**WT**	**V33D**	**V33G**	**V33Y**
Alanine	2.4 ± 0.5	1.28 ± 0.08	1.01 ± 0.06	1.98 ± 0.08
Valine	0.427 ± 0.003	1.03 ± 0.03	0.60 ± 0.02	0.025 ± 0.003
Leucine	0.34 ± 0.01	0.51 ± 0.03	0.190 ± 0.005	0.23 ± 0.01
Methionine	1.52 ± 0.06	1.28 ± 0.02	0.9 ± 0.1	1.03 ± 0.03
Serine	0.86 ± 0.01	0.18 ± 0.01	0.073 ± 0.003	0.34 ± 0.01
Glutamine	1.5 ± 0.1	1.03 ± 0.02	0.54 ± 0.03	0.89 ± 0.03
Arginine	0.80 ± 0.08	0.51 ± 0.02	0.19 ± 0.02	0.35 ± 0.01
Lysine	Nd[a]	Nd	Nd	Nd
Histidine	0.07 ± 0.01	0.12 ± 0.02	0.046 ± 0.005	0.084 ± 0.002
Phenylalanine	0.100 ± 0.002	0.59 ± 0.02	0.28 ± 0.01	0.47 ± 0.03
Phenylglycine	Nd	0.014 ± 0.001	Nd	Nd
O-methyl-tyrosine	0.091 ± 0.008	0.124 ± 0.001	0.041 ± 0.005	0.064 ± 0.002
4-nitro-phenylalanine	0.040 ± 0.005	0.076 ± 0.003	0.032 ± 0.003	0.075 ± 0.002
3-methyl-phenylalanine	0.146 ± 0.006	0.82 ± 0.02	0.51 ± 0.02	0.42 ± 0.04
4-methyl-phenylalanine	0.107 ± 0.007	0.124 ± 0.004	0.042 ± 0.004	0.096 ± 0.001
Tryptophan	0.205 ± 0.006	0.149 ± 0.009	0.058 ± 0.003	0.016 ± 0.003

[a] No activity was detected.

The use of bulky aliphatic amino acids results in lower specific activity for the wild type. As shown on Table 3.4, wild type DAAT has an approximately 6-fold lower specific activity with D-valine (0.427 ± 0.003 U/mg) and a 7-fold lower specific activity with D-leucine (0.34 ± 0.01 U/mg) than with D-alanine. On the other hand, V33D and V33G display higher specific activities than wild type with D-valine (2.4- and 1.4-fold, respectively). This result is in agreement with a previous study that reported a significantly increased affinity of V33A for α-ketoisovalerate, the α-keto acid of valine, compared to the wild type [20]. The authors suggested that this result may be due to the replacement of the Val residue at position 33 by a smaller one (Ala), enabling reactivity with a bulkier substrate. Although the V33D and V33G mutants displayed increased activity with D-valine compared to wild type, it was still not as high as their activity with native substrate D-alanine. In addition, the V33G mutant is 1.8-fold less active with D-leucine than wild-type DAAT. This indicates that mutation of V33 to smaller residues is not necessarily sufficient for accommodation of bulky aliphatic substrates. Interestingly, both V33G and V33D have a specific activity with D-methionine that is within error to that obtained with D-alanine. Although D-methionine is much bulkier than D-alanine, it is the most conformationally flexible amino acid, which may allow it to fit in binding pockets of different sizes and shapes. Of note, the V33Y mutant has lower activity than wild type with both D-valine and D-leucine, while its activity with D-methionine is lower than with D-alanine. This may be due to steric clashes between the large aromatic side-chain of the tyrosine residue and the bulky aliphatic side-chains of these substrates.

We also tested various neutral and charged polar amino acids. All of these amino acids, with the exception of D-lysine, resulted in lower specific activities for the mutants

relative to the wild type. Our data shows that D-lysine is not a substrate of DAAT and its mutants, a result in agreement with a previously published report from our group using the MINISEP-MS assay [19]. Interestingly, D-glutamine is a better substrate of wild type DAAT and its mutants than the smaller D-serine, supporting our observation that there is no direct correlation between the size of the substrate and the residue at position 33.

We were most interested in assaying the activity of DAAT and its mutants with aromatic amino acids, as we expected these mutants to be significantly more active than wild type for these substrates. As expected based on our previous results with the DAAO assay, all three mutants were more active than wild type with D-phenylalanine as a substrate. DAAT V33D was the most active at 0.59 ± 0.02 U/mg, approximately 6-fold more active than wild type. V33G and V33Y were 2.8- and 4.7-fold more active than wild type with D-phenylalanine, respectively, a result in good agreement with our previous data [10]. We then evaluated whether our DAAT mutants also displayed improved activity with the smaller aromatic amino acid, D-phenylglycine. Only V33D was active with D-phenylglycine with a specific activity 91-fold lower than with D-alanine, indicating that it is a very poor substrate. This result can be explained by the structure of the DAAT active site. As shown in Figure 3.6, the methyl side-chain of D-alanine is directed towards a loop formed by residues S240-T241-T242-S243. Although the methyl side chain of D-alanine can fit in this restricted space, the phenyl side chain of D-phenylglycine cannot, which explains why this amino acid, which is smaller than D-phenylalanine, is either not a substrate or a very poor one. On the other hand, D-phenylalanine can be bound by DAAT because its phenyl ring is attached to a methylene group, allowing a conformation that positions the phenyl ring away from this loop in a small cavity next to the side chain of V33. D-Phenylglycine, lacking this methylene

group, is forced to have its phenyl ring pointing towards the loop, causing steric clashes. Based on the DAAT structure, it is expected that D-phenylglycine would not be a substrate of DAAT or any of the mutants tested, an observation in agreement with our kinetic data.

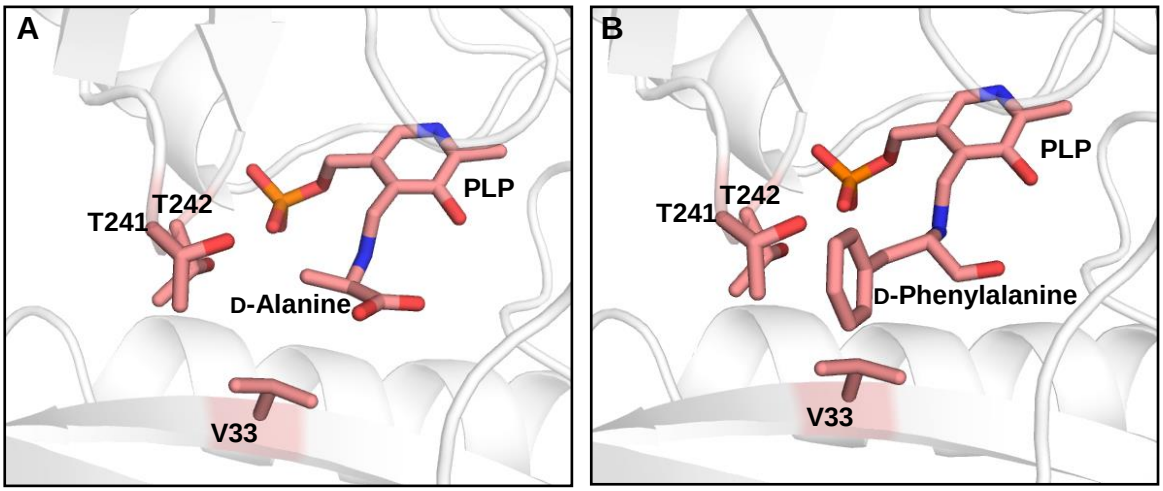

Figure 3.6 DAAT Active Site. (A) The crystal structure of the active site of *Bacillus* sp. YM-1 DAAT with bound PLP and D-alanine substrate (PDB ID 3DAA) is shown in cartoon representation. The PLP-bound D-alanine substrate along with residues V33, T241, and T242 are shown in sticks. The D-alanine side-chain points towards the loop including residues T241 and T242. (B) D-Phenylalanine was modelled in the active site of DAAT in place of D-alanine using PyMOL. The methylene group of the D-phenylalanine side-chain allows for a conformation in which the phenyl ring points away from T241 and T242.

Knowing that all of our mutants were more active with D-phenylalanine than the wild type enzyme, we decided to test derivatives of this amino acid to investigate which types of substitutions on the aromatic ring would be tolerated by our mutants. Of the derivatives tested, 3-methyl-D-phenylalanine was the most active with both the wild-type and mutant enzymes. The wild type is 1.5-fold more active with 3-methyl-D-phenylalanine than D-

phenylalanine, and V33D, V33G, and V33Y are 5.6-, 3.5-, and 2.9-fold more active than wild type with 3-methyl-D-phenylalanine, respectively. The fact that all mutants are significantly more active than wild type with this substrate indicates that there is room in the binding pocket to accommodate a methyl group in the *meta* position. Furthermore, both V33D and V33G are 1.4- and 1.8-fold more active with 3-methyl-D-phenylalanine than D-phenylalanine, an observation that suggests that the 3-methyl group allows for additional stabilizing interactions of the substrate in the active site. Conversely, all of the other D-phenylalanine derivatives tested involved substitutions in the *para* position, and it is apparent that substitutions at this position are not well accommodated by the wild type or the mutant enzymes (Table 3.4). With 4-methyl-D-phenylalanine, wild type, V33D, and V33Y had activity similar to wild type activity with D-phenylalanine, while V33G displayed lower activity. Similar trends were observed with *O*-methyl-D-tyrosine and 4-nitro-D-phenylalanine as substrates. This leads to the conclusion that although the V33D, V33G, and V33Y mutants are better than wild type at accommodating the large D-phenylalanine substrate, enough space has not been created in the active site to allow for a substituent in the *para* position.

Measuring specific activity values for DAAT with a variety of substrates has provided valuable information regarding the enzyme's specificity. However, more data is needed to fully understand the causes of DAAT wild type and mutant substrate preference. As specific activity values are measured with a fixed substrate concentration, the results are highly condition-dependent. For example, it is possible that the D-amino acid concentration used (10 mM) for these assays is below the K_M for the enzyme, resulting in rates lower than vmax. To characterize in more detail the substrate specificity of DAAT and its mutants, the apparent kinetic parameters (K_M and k_{cat}) should be measured using the DDO assay.

Additionally, although we have found that our mutants display increased activity towards the aromatic substrates D-phenylalanine and 3-methyl-D-phenylalanine, the highest activity observed (0.82 ± 0.02 U/mg, V33D with 3-methyl-D-phenylalanine) is still approximately 3-fold lower than the activity of the wild-type enzyme with its native substrate D-alanine. Therefore, further rounds of engineering are necessary to increase the activity of these mutants towards aromatic amino acids. Nevertheless, the V33D, V33G, and V33Y mutants are promising leads for the development of efficient biocatalysts for the asymmetric synthesis of these desirable compounds.

3.4 Conclusion

We have developed a coupled assay for DAAT activity using DDO as a coupling enzyme, allowing for the detection of DAAT activity with a broad range of D-amino acids. Using this assay, we have further characterized the substrate specificity of the DAAT mutants V33D, V33G, and V33Y, previously discovered to have increased activity with the non-native substrate phenylpyruvate. All three mutants were more active than wild-type with D-phenylalanine and 3-methyl-D-phenylalanine, while V33D and V33G were more active than wild type with D-valine. Therefore, we have shown that these mutants allow for increased activity with some aromatic and bulky aliphatic amino acids, warranting further investigation and engineering for use as biocatalysts for production of these valuable chemicals.

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4. Conclusions and perspectives

D-Amino acids are essential building blocks in the pharmaceutical industry (Chapter 1). In order to synthesize these compounds in enantiopure form, biocatalysis can be used as enzymes display exquisite stereospecificity. However, due to the relative rarity of D-amino acids in nature compared to L-amino acids, there are few naturally-occurring enzymes that produce D-amino acids, limiting the development of biocatalytic methods for the synthesis of these compounds. While methods for the biocatalytic production of D-amino acids are available, these have drawbacks such as the need to provide expensive D-amino acid or α-keto acid substrates and limited substrate scope [1]. The focus of this work has been D-amino acid aminotransferase (DAAT), whose use as a biocatalyst for the production of enantiopure D-amino acids has been documented [2, 3, 4, 5, 6]. While DAAT can synthesize a variety of D-amino acids, it has relatively low activity with aromatic and branched chain D-amino acids. Therefore, in order to improve established methods of D-amino acid synthesis using DAAT, protein engineering is required to create variants of DAAT that are more active towards additional D-amino acids.

One of our goals was to create DAAT mutants with altered substrate specificity using protein engineering techniques. A key component of protein engineering is a method for the detection of active mutants. Ideally, this method should be sensitive, high-throughput, and versatile. We found that an ideal method for the screening and characterization of DAAT mutants did not exist. Therefore, we developed the DAAO coupled assay [7] for screening large libraries of DAAT mutants. Other assays available for the high-throughput detection of DAAT activity utilize coupling enzymes such as lactate-dehydrogenase (LDH) and L-glutamate-dehydrogenase (GDH) [8]. While useful, these assays detect the α-keto acid

product of DAAT (pyruvate or α-ketoglutarate for the LDH and GDH assays, respectively) instead of the D-amino acid product. In addition, methods that allow for direct detection of the D-amino acid product involve separation via capillary electrophoresis or HPLC followed by mass spectrometry detection, resulting in decreased throughput. Therefore, the DAAO coupled assay that we developed is unique in that it is a high-throughput method that detects the formation of the D-amino acid product of the transamination reaction catalyzed by DAAT. It can thus be used to confirm the stereospecificity of mutant DAAT enzymes, which is important as mutagenesis to alter substrate specificity may unintentionally result in the alteration of stereoselectivity. We were also able to decrease costs and increase throughput by voiding the need to purify or purchase DAAO for screening purposes, as it was co-expressed with DAAT in a pETDuet vector. We demonstrated the sensitivity of the DAAO assay by detecting the transamination activity of the Y31R mutant, a mutant approximately 2,000-fold less active than wild type whose activity could not be detected using the GDH coupled assay. Finally, we were able to use the DAAO assay for its intended purpose – identifying DAAT mutants with altered substrate specificity.

A useful aspect of the DAAO coupled assay is the fact that many D-amino acids can be detected, allowing us to screen mutant DAAT libraries for increased activity with a wide range of non-native substrates. As described above, we were interested in engineering DAAT for increased activity towards aromatic substrates. Using the DAAO coupled assay, we successfully identified three mutants – V33D, V33G, and V33Y – that are more active than wild type with the non-native substrate phenylpyruvate. The two mutants that were kinetically characterized, V33G and V33Y, have k_{cat}/K_M values that were approximately 3-fold higher than that of the wild type for phenylpyruvate. While these improved mutants are

not as efficient at producing D-phenylalanine as the wild type is at producing D-glutamate, a 3-fold increase in activity is still significant from a biocatalysis perspective. For example, if one of these mutants was to be incorporated into one of the biocatalytic processes described in Chapter 1.4, it would enable shorter reactions requiring lower amounts of biocatalyst to yield similar amounts of D-phenylalanine than those produced with the wild-type enzyme. Overall, this would translate into a lower cost for the synthesis of this compound. However, to improve efficiency, further rounds of engineering would be carried out in order to create DAAT mutants that are as or more active towards non-native substrates than the wild type enzyme is with its native substrates D-alanine and D-glutamate.

We also developed a coupled assay to enable the detection of DAAT activity with a broad range of D-amino acid substrates. As most α-keto acids of interest are unavailable commercially, screening mutants with a D-amino acid of choice is more practical in some cases. The DDO coupled assay was used to profile the specific activity of the DAAT V33D, V33G, and V33Y mutants with a broad range of D-amino acids. Our results obtained with the DDO assay confirmed that all three mutants are indeed more active than wild type with D-phenylalanine, but other interesting results were obtained as well. None of the mutants were particularly active with D-phenylalanine derivatives that had substitutions in the *para* position of the phenyl ring, yet all three mutants were as or more active with 3-methyl-D-phenylalanine than with D-phenylalanine. Futhermore, V33D and V33G were more active than wild type with D-valine, a branched chain amino acid. These results illustrate the usefulness of the DDO assay, which allowed us to characterize the scope of D-amino acids that are substrates of wild-type and mutant DAATs. To gain additional information on the substrate specificity of mutant DAATs, these enzymes can be further characterized by

measuring their apparent kinetic parameters for D-amino acid substrates using the DDO assay.

The DAAO and DDO coupled assays developed herein are broadly applicable, and could be further improved and modified. The DDO coupled assay was limited by the lower specific activity of DDO. Thus, the DDO coupled assay was not optimized for high-throughput screening of mutant libraries using a co-expression system. In order to improve the DDO coupled assay and expand its possible implementation, DDO could be engineered for increased activity using a method such as directed evolution. High-throughput screening of DDO mutants could be carried out with a colorimetric assay using horseradish peroxidase as a coupling enzyme. Together with the DAAO assay, an optimized DDO assay could thus be used to screen mutant libraries of any enzyme that produces D-amino acids, such as the stereo-inverting enzyme D-phenylglycine aminotransferase. With these assays, our group is now able to engineer any aminotransferase for the production of desired unnatural D-amino acids.

The DAAT V33D, V33G, and V33Y mutants displaying increased activity toward D-phenylalanine described in this work were obtained after one round of rational design. A further round of rational design that is presently underway in our group involves the mutagenesis of interdomain loop residues P199, R120, and P121. Mutation of these residues to glycine has been previously shown to increase activity with D-phenylalanine by 3-fold [9]. We hypothesized that the combination of this triple mutation motif with the V33D/G/Y mutations will result in DAAT variants displaying greater activity towards D-phenylalanine and other aromatic D-amino acids. In addition, we are interested in solving the crystal structure of the V33 mutants in order to aid in identifying other residues and mutations that

could contribute to increased D-phenylalanine activity. Finally, we will use the three DAAT mutants identified herein as starting points for further rounds of engineering by other means, such as directed evolution or computational protein design, in order to create improved biocatalysts for the production of any desired D-amino acid.

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