<u>Crystallisation and Structural Studies on</u> <u>Omega Transaminase Enzymes</u>

Submitted by

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Abstract

Omega transaminase enzymes are proving to be of vital importance in the production of chiral amines for drug manufacturing due to their high stereoselectivity and regioselectivity reactions. Omega transaminases also possess another key feature that can make them more desirable than other transaminase enzymes in that they are able to utilise substrates lacking a carboxyl group.

This project has purified several recombinant omega transaminases from the bacteria; *Streptomyces avermitilis*, *Deinococcus geothermalis*, *Pseudomonas putida* and *Pseudomonas aeruginosa*. Attempts have been made to crystallise the proteins in order to determine their structures. *D. geothermalis* omega transaminase failed to purify due to lack of binding to Nickel columns despite having a histidine tag. *P. putida* omega transaminase failed to produce any crystals in the crystal screens available. *P. aeruginosa* omega transaminase crystals, whose holoenzyme structure has already been solved, were soaked in pyruvate and also produced crystals from co-crystallisation experiments with pyruvate. These crystals however did not show sufficient electron density in the active site to determine if the substrate had bound. They did however diffract to 2.0 Å and 1.8 Å resolution respectively. Co-crystallisation experiments with *P. aeruginosa* omega transaminase and the inhibitor gabaculine were successful however to date they have failed to produce X-ray diffraction data that could be processed despite diffracting to high resolution.

The *S. avermitilis* omega transaminase produced crystals that were shown to be disordered when they underwent X-ray diffraction. Differential scanning fluorometry was carried out to identify the optimal buffer for protein stability. This identified the current Tris pH 7.5 buffer to lead to a low thermodynamic stability of the protein. Several new buffers including MES at pH 6 were identified as being the most likely candidates for stabilising the protein and producing ordered crystals.

Data collected from a *Chromobacterium violaceum* omega transaminase with a F89 to A89 mutation was successfully processed and refined. The resultant structure showed an increase in flexibility in the substrate carboxyl binding residue, R416, and an increase in space available for substrate binding. HPLC assays showed alpha-methylbenzylamine activity remained. An amino acid oxidase/horseradish peroxidase linked assay showed there to be a small increase in activity towards β -alanine due to this mutation. Further mutations will be necessary to enable this enzyme to utilise β -alanine at a more practical rate of conversion.

Abbreviations

4-AAP	4-Aminoantipyrine
AcornAT	Acetylornithine aminotransferase
AlaAT	Alanine aminotransferase
AP	Acetophenone
APS	Ammonium persulfate
AT	Aminotransferase
AspAT	Aspartate aminotransferase
BcaaAT	Branched chain amino acid aminotransferase
Da	Daltons
DaaAT	D-amino acid aminotransferase
D-alaAT	D-alanine aminotransferase
DapaAT	7,8-Diaminopelargonic acid aminotransferase
dd	Double distilled
EPPS	4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid
GabaAT	γ-Aminobutyric acid aminotransferase
GF	Gel filtration
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HisPAT	Histidinol-phosphate aminotransferase
His-tag	Poly histidine tag
IPTG	Isopropyl β-D-galactopyranoside
MBA	α-methylbenzylamine
MES	4-Morpholineethanesulfonic acid
MOPS	4-Morpholinepropanesulfonic acid
mPMS	1-Methoxy-5-Methylphenazinium Methyl Sulfate
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
OrnAT	Ornithine aminotransferase
PAGE	Polyacrylamide gel electrophoresis
PDB	Protein Databank
PheAT	Phenylalanine aminotransferase
PIPES	1,4-Piperazinediethanesulfonic acid
PLP	Pyridoxal-5'-phosphate
PMP	Pyridoxamine phosphate
PSerAT	Phosphoserine aminotransferase
SDS	Sodium dodecyl sulfate
SerAT	Serine aminotransferase

TBHBA	2, 4, 6-Tribromo-3-hydroxybenzoic acid
TEMED	N,N,N,N-tetramethylethylene diamide
Tris	Tris[hydroxymethyl]aminomethane
TyrAT	Tyrosine aminotransferase
ω-AaAT	ω-amino acid aminotransferase

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Chapter 1 - Introduction

1.1. Applications of Biocatalysis

Recently enzymes are being utilized as biocatalysts in a myriad of industrial processes for their high specificity, consistent results and, unlike tradition manufacturing materials, usually involving high temperatures, high pressures, toxic transition metals or a combination of the three, are reusable and can be highly stereoselective [1]. Examples of biocatalytic processes include the breakdown of amides by penicillin acylase, hydrolysis of esters to acids by esterases and lipases, the formation of malic acid by condensation by fumarase, conversion of glucose to fructose by glucose isomerase and the removal of a halogen by dehalogenases [2]. Many semi-synthetic antibiotics are now being made by utilising biocatalysis instead of chemical conversions based on stoichiometry. α -amino acid ester hydrolase, α -acylamino- β lactam acylhydrolase and ampicillin acylase are all enzymes that have been reported capable of producing semi-synthetic cephalosporins from 7-aminocephem or 6aminopenam with α -amino acid esters [3]. The immobilized enzyme penicillin acylase is being used in the manufacturing process of β -lactam antibiotics. Condensation of 6aminopenicillanic acid with D-(-)-4-hydroxyphenylglycine or D-(-)-phenylglycine by this enzyme will make amoxicillin or ampicillin respectively. Also cefadroxil and cephalexin can be made from 7-aminodesacetoxycephalosporanic acid in the same manner [4]. Whole cells can be used in complex reaction pathways that require regeneration of co-factors for the enzymes as it is far easier and cheaper to allow a living cell to do all the work in ensuring the enzyme remains active [1]. Due to their high specificity, there are few by-products of enzyme based reactions which with increasing environmental awareness becomes even more of a necessity as any waste must be dealt with properly, so the less there is the cheaper the end product. This specificity translates into the chirality of the end product which in drug manufacturing is vital as demonstrated by the dispute over a failed drugs test in the 2002 winter Olympics. Alain Baxter tested positive for amphetamines and subsequently lost his bronze medal, caused by a Vick's nasal spray which contained a legal isomer, Lmethamphetamine, of the banned drug D-methamphetamine, which shows how much biological interactions rely upon chirality [5]. In a review by Ramesh N. Patel 2002 [6], the importance of chiral intermediates for drug manufacturing is highlighted with examples such as chiral amino acid intermediates for the production of β3-Receptor agonists by an amidase from *Mycobacterium neoaurum*. HIV protease inhibitors are effective in combating retroviral infection. One of their synthesis steps requires the transamination of 1-keto-2-hydroxyindan to cis-1-amino indanol as shown in Fig 1.1. [6]. Production of inhibitors for matrix metalloprotease also require optically pure intermediates. These are used for the treatment of both cancer and rheumatic inflammation [7].



Indinavir (Crixivan)

Fig. 1.1. Synthesis pathway of HIV protease inhibitor including the transamination reaction modified from Patel, 2002 [6].

1.2. Transaminase Enzymes - Background

Transaminases (aminotransferases) traditionally have been used as an indicator in medical tests for liver disease to determine how much cell death is occurring within the liver. This has been useful in diagnosing hepatitis infection and liver disease from factors such as excessive alcohol consumption. It is also vital in the glucose-alanine cycle where transamination of pyruvate into alanine in the muscles for transport in the blood to the liver where it is converted back into pyruvate and then into glucose to be used by the energetically active muscle cells. Lately however transaminases are being seen as useful biocatalysts in industrial processes to manufacture optically pure amines. As their name suggests, they transfer amino groups from a donor containing an amine group, to a ketone acceptor which is the method by which the body creates what is known as the 'non-essential amino acids'.

Recently a transaminase was designed by Merck and Codexis for the production of sitagliptin, the active component of Januvia which is used in the treatment of type 2 diabetes [8]. Prior to its development, sitagliptin was produced by enamine formation, followed by asymmetric hydrogenation at 250 pounds per square inch with a rhodium-based catalyst. This resulted in a mixture of sitagliptin at 97 % enantiomeric excess with a trace of rhodium. Recrystallisation was needed to improve the enantiomeric excess followed by addition of H₃PO₄ to produce sitagliptin phosphate. Homology modelling of the transaminase was used for the initial hypothetical library designs. Docking studies showed the (R)-selective transaminase ATA-117 to be incapable of binding the prositagliptin ketone due to steric interference in the small binding pocket and undesired interactions in the large binding pocket. Directed evolution was used along with substrate walking and point mutations based on the model to produce an enzyme containing a total of 27 mutations that was capable of the amination of prositagliptin ketone to sitagliptin at 99.95 % enantiomeric excess thereby removing the need for a rhodium catalyst and high pressure in the reaction step. Also recrystallisation is no longer required as it is already enantiopure [8]. This is a prime example of how transaminases and knowledge of their structure can prove useful in the pharmaceutical market. For this Merck and Codexis were awarded the Greener Reaction Conditions award in 2010.

1.3. Transaminase Mechanism

The co-factor for transaminase enzymes is pyridoxal-5'-phosphate (PLP), known as vitamin B6. It is used in a variety of catalytic reactions apart from transamination including decarboxylation and racemization. PLP enzymes are so versatile that it is estimated that 1.5 % of the total open reading frames in bacterial genomes encodes PLP dependant enzymes [9]. The enzyme forms an internal aldimine with the ε -amino group of a lysine in the active site and is capable of accepting electrons from the substrate. During the enzyme-substrate complex reaction (Fig. 1.2.) the lysine is replaced by the amino group of the amino donor which then forms an external aldimine [10]. Dunathan [11] hypothesised that the next step in the reaction was dependant upon which of the three bonds to the α carbon were cleaved, the scissile bond of the external aldimine has to be perpendicular to the plane of the π system bond between the coenzyme and the donor due the low activation energy of this particular configuration, forming a carbanion intermediate. The α -hydrogen is then

taken away from the amino acid donor to form a planar intermediate before being transferred to the C4 atom of the PLP molecule. The intermediate is then hydrolysed to pyridoxamine 5-phosphate (PMP) and a keto acid. The second step of the reaction involving the amino acceptor keto acid and the regeneration of PLP involves the exact same process working backwards [12]. It is agreed that the mechanism of transaminase reaction is by the 'bi ping-pong' system. Aspartate aminotransferase has been studied in more detail than any other transaminase and provides a good example. Its substrates and products oxaloacetate, aspartate, 2-oxoglutarate and glutamate bind to the enzyme through binding between Arg-386 and Arg-292 and their α - and ω -carboxyl groups via salt bridges and hydrogen bonding [13].



Fig. 1.2. The transamination reaction involving pyridoxal-5'-phosphate cofactor and its intermediates modified from Soda, 2000 [12].

1.4. Transaminase Classification

The transaminases can be split up into four sub-groups, according to Mehta et al (1993) [14] by comparing sequence homology, predicted secondary structure and hydropathy patterns for all the transaminase enzymes known at the time (Table 1.1). Sub-group I contains the transaminases AspAT, AlaAT, TyrAT, HisPAT and PheAT and overall this group seems to have a preference for 2-oxoglutarate as the keto acid substrate. Group III contains D-AlaAT and BcaaAT and both exhibit a preference for 2-oxoglutarate as the keto acid substrate. D-AlaAT is a necessary enzyme for any bacteria possessing a cell wall as it is the mechanism by which D-alanine and Dglutamate are made which are important cell wall components [15]. Group IV includes SerAT and PSerAT and they use L-serine and 3-phospho-L-serine as amino acid donors respectively. However it is the group II that this project will be focusing on, containing members such as AcornAT, OrnAT, WAaAT, GaBaAT and DapaAT which are known as omega-aminotransferases [14]. These are so called due to the fact that during the first half of the reaction there is a transfer of an amino group to the position distant to the α carbon. This will always occur even when there is the option of selecting a second amino group on the α group with the same configuration, although during the second half reaction there is an α -transamination like most other aminotransferases [16] [17].

Mehta sub-groups	Transaminase Members	Pfam sub-groups
Ι	Aspartate + Aromatic transaminase	I + II
II	Omega transaminase	III
III	Branched chain transaminase	IV
IV	Phosphoserine transaminase	V
-	ArnB transaminase	VI

Table 1.1. Comparison between the Pfam and Mehta sub-groups. The main transaminase members for each group are listed in the second column with the assigned groups from Mehta [14] in the first column and from Pfam in the third [14][18][19]. Mehta subgroups I, III and IV (Pfam subgroups I, II, IV, V and VI) are classified as alpha transaminases which catalyse amino donors with an adjacent carboxyl group. Mehta subgroup II (Pfam subgroup III) are classified as omega transaminases, which do not necessarily require a carboxyl group on the amino donor.

Recently, transaminase enzymes have been reclassified into six sub-groups according to their structure by the Pfam database [18]. All sub-groups of transaminases belong to the same fold family. The exception to this is group IV, which belongs to a different fold family and includes members such as branched chain transaminases. Omega transaminases were assigned to group III, while alpha transaminases were assigned to the other five groups. Aspartate and aromatic transaminases were assigned to group V and ArnB transaminases were assigned to group VI [19].

1.5. Omega Transaminase Specificity

Unlike all previously mentioned subgroups of transaminases, these can catalyse a reaction between a keto acid and an amino donor with the absence of a carboxyl group from the latter, giving rise to more options for biocatalysis. *Vibrio fluvialis* JS17 possesses a novel omega transaminase which has been under much investigation and has shown it to have broad substrate specificity and it has high selectivity for (S)-enantiomers. It catalyses the reaction of (S)- α -methylbenzylamine (MBA) and pyruvate to acetophenone (AP) and L-alanine (Fig. 1.3). The reaction provides a way to quantitatively measure the activity of the enzyme as the equilibrium lies heavily on the side of the products [20].



Fig. 1.3. Conversion of (S)- α -MBA and pyruvate to acetophenone and L-alanine by omega transaminase. The equilibrium of the reaction favours the right hand side [20].

Generally speaking, omega transaminase enzymes belong to a family of β -Alanine:pyruvate transaminases where the preferred amino acceptor substrate is pyruvate not α -ketoglutarate [20]. The omega transaminase from *Pseudomonas aeruginosa* was revealed to be capable of aminating the aliphatic ketodiol l-erythrulose [21].

In a report by Ward *et al* 2007 [20], it was shown that the omega transaminase obtained from *Chromobacterium violaceum* did not possess enantioselectivity towards either (R)- or (S)-1,3-dihydroxy-1-phenylpropan-2-one as substrates but was highly (S)-stereoselectivity in the conversion to 2-amino-1-phenyl-1,3-propanediol with 99 % of the resulting mixture in the (S) form [22]. This allows a chiral product to be made from a non-chiral mixture which is far more cost effective.

C. violaceum omega transaminase was shown to possess high activity towards aromatic (S)-amines with the best donors of those tested, 1-aminoindane, 1-methyl-3-phenylpropylamine and benzylamine. Currently there appears to be no activity towards aminodiols by this enzyme. A difference is observed in the initial rates of reaction with the (S)-enantiomers of MBA and 1-aminoindane when compared to the racemic mixtures of these substrates [20]. This differs to *V. fluvialis* omega transaminase where a racemic mixture resulted in a lower activity, thought to be the result of inhibition by (R)-enantiomers [23]. As for acceptors, aliphatic and aromatic aldehydes both show good activities for *C. violaceum* omega transaminase with keto acids being the one group that was shown to have low activity. Both *V. fluvialis* and *C. violaceum* omega transaminases can only utilise L-alanine as an amine donor out of the range of α -amino acids tested. Overall it has been shown that *C. violaceum* omega transaminase has a broad substrate range, especially where amine acceptors are concerned [20].

1.6. Omega Transaminase Structure

The crystal structure of *C. violaceum* omega transaminase was solved by Dr Christopher Sayer (Fig 1.4.) [24]. In general transaminases form homodimers with an active site consisting of two binding pockets. Each of these subunits consists of a large domain where the PLP is bound, made up of a pair of α helices on either side of a seven stranded β sheet and a small C-terminal domain. This has a small β sheet with α helices around it. There are several essential amino acid residues in the active site that are responsible for vital interactions (Fig. 1.5.). An aspartic acid at position 259 is needed to form a hydrogen bond with the PLP, the active site lysine that interacts with the PLP as described earlier is at position 288 and an arginine which forms a saltbridge with α -carboxylate of the substrate is located at position 416 (all numbers refer to *C. violaceum* CV2025 sequence) [20]. In the large substrate binding site of the omega transaminase from *V. fluvialis* is a pair of tryptophan residues, W57 and W147, that control substrate specificity by restricting the active site due to their large side chains (Fig. 1.6.). Mutation of these to glycine residues results in increasing the range of substrates they accept, allowing them to catalyse the transamination of aliphatic amines without losing the original activity of the enzyme towards aromatic amines and its enantioselectivity. This suggests that loss of these residues allows increased flexibility in the large substrate binding site. Also in the ornithine transaminase complexed with an inhibitor, it has been shown that the glutamic acid E235 can mask the conserved tyrosine residue, needed to bind the substrates α -carboxylate, via hydrogen bonding and salt bridges [25].



Fig. 1.4. Tertiary structure of the holoenzyme of *C. violaceum* omega transaminase dimer viewed normal to the molecular two-fold axis, produced by PyMOL [26] (PDB code 4AH3). PLP cofactor is represented by spheres and lighter shaded domains are the small domains [24].



Fig. 1.5. Key residues are shown here represented as stick models using PyMOL [26]. Dashed line between Asp259 and PLP represents the hydrogen bond (distance 2.94 Å). PLP is bound to Lys288. Right is Arg416.



Fig. 1.6. Trp57 and Trp157 are shown here with PLP located between them using PyMOL [26]. Large side chains restrict the active site.

1.7. Inhibition of Omega Transaminases

Both α and ω transaminases are vulnerable to the suicide inhibitor gabaculine (Fig. 1.7.). This compound acts as a neurotoxin in the body, capable of crossing the bloodbrain barrier and inhibiting GABA-AT and allowing GABA, an inhibitory neurotransmitter, to rise significantly [27]. After binding to the active site a Schiff base is formed with the PLP and a proton is removed from the β -carbon bound to the imino group as would happen with a normal amino donor substrate. However with gabaculine a second proton is taken from the β -carbon, this creates an unstable intermediate that then goes on to form m-carboxyphenylpyridoxamine phosphate (mCPP), this is very stable and as such is irreversible preventing PLP from being formed again [28][29]. The mechanism is displayed in Fig. 1.8.



Fig. 1.7. Molecular structure of the inhibitor gabaculine [30].



Fig. 1.8. Mechanism of inhibition of transaminase cofactor PLP by gabaculine modified from Fu and Silverman, 1999 [30].



Fig. 1.9. Gabaculine bound to PLP to form mCPP [24] within the active site drawn using PyMOL [26].

1.8. Differential Scanning Fluorometry Theory

Protein crystallisation results can sometimes be dependant upon the stability of a protein. Disordered proteins crystals can be caused by loose floppy domains which prevent proteins packing into ordered crystals. A way around this is to attempt to optimise the conditions where the protein is most stable and hence reduce the number of different conformations the protein is present in. To achieve this Gibbs free energy of unfolding (G_u) must be taken into consideration. This is determined enthalpy (H) and entropy (S): G = H - TS. This means that as temperature increases and the protein begins to unfold and gain entropy, G_u begins to decrease. At $G_u = 0$, an equilibrium is reached, this is called the Tm where half of the protein is in the folded state and the other half in the unfolded state. Therefore a higher Tm value is indicative of a more stable protein [31].

Differential scanning fluorometry (thermal shift assay) allows the determination of a more thermodynamically stable protein. A number of factors such as buffers, salts, pHs' and ligands can be altered and their effects upon the stability of the protein measured. Sypro orange is used as a indicator of denatured proteins as it bind to hydrophobic regions, which under normal circumstances in cytosolic proteins are buried until exposed due to unfolding of the protein. Upon binding there is an increase in fluorescence at a wavelength of 610 nm with an excitation wavelength at 492 nm,

which can be measured on a quantitative PCR machine [31]. Figure.1.10. shows how this works [32]. This allows rapid screening of different conditions in which to stabilise the proteins or the stabilisation effect of various compounds when they bind to the protein. This technique can sometimes prove invaluable when disordered protein crystals are a common result of crystallisation trials.



Figure. 1.10. Visualisation of the correlation between protein unfolding and binding of Sypro orange. Y axis displays fluorescence of Sypro orange, X axis displays temperature. Image N shows protein in its natural state with hydrophobic regions displayed as grey areas buried. As the temperature increases the protein begin to unfold as is shown by image U, Sypro orange binds to the hydrophobic regions and fluoresces(orange lines). Figure A shows the denatured proteins aggregating together and the amount of available hydrophobic sites for Sypro orange binding decreasing. Tm is shown on the image as being halfway between the natural and denatured states which corresponds to halfway between baseline and the peak of the curve. Figure taken from Niesen *et al* 2007 [32].

1.9. Previous Work Relevent To This Project

1.9.1. Structural Studies of Native Omega Transaminases

The crystal structure of *P. aeruginosa* omega transaminase (Fig. 1.11.) has been previously solved [24]. Only the holoenzyme of this was resolved, no structure has been obtained in complex with substrate or gabaculine bound in the active site.



Fig 1.11. Tertiary structure of the holoenzyme of *P. aeruginosa* omega transaminase dimer viewed normal to the molecular two-fold axis, produced by PyMOL [26]. Black sticks represent PLP and lighter coloured domains are the small domains [24].

1.9.2. Site Directed Mutagenesis of Omega Transaminases

A mutant of *C. violaceum* omega transaminase was previously made (work to be published) with a F89 \rightarrow A89 substitution. This was done as part of a group of mutants that were produced through site directed mutagenesis to analyse the effects they had on the activity of the enzyme towards β -alanine to which the native enzyme has no activity towards. The structure of *P. aeruginosa* omega transaminase which uses β -alanine as a substrate [21] was analysed and compared to the *C. violaceum* omega transaminase to determine differences that might cause this difference in substrate specificity. Substrates such as β -alanine are restricted by their carboxyl group. Arg416 is responsible for binding the carboxyl group of substrates, whose movement is limited by Phe89 in the nearby subunit. After the Arg416 binds β -alanine, the Phe89 prevents the Arg416 assuming an orientation where the nitrogen group of β -alanine is in the catalytic position (Fig. 1.12.). The Phe89 was mutated into an Ala89 in the hope that the loss of the aromatic group will result in a more flexible active site which will allow β -alanine to be used as a substrate.



Fig. 1.12. The Phe89 residue is shown positioned near to Arg416 in the *C. violaceum* omega transaminase [24] (PDB code 4AH3), possibly preventing it from moving into the catalytic orientation after binding β -alanine. Image made using PyMOL [26].

Aims of Project

The main objective of this project was the purification of *Streptomyces avermitilis*, *Pseudomonas putida* and *Deinococcus geothermalis* omega transaminase enzymes and to determine their structures through crystallographic methods. None of these omega transaminases currently has a crystal structure determined to date. Previous work done on the *S. avermitilis* omega transaminase enzyme has resulted in successful protein crystals however none have produced viable data as all have been disordered.

An aim was to also co-crystallize *P. aeruginosa* omega transaminase in complex with the inhibitor gabaculine and in complex with the substrate pyruvate.

Cloned omega transaminase enzymes will be over-expressed and purified using Nickel affinity and gel filtration chromatography where possible. Crystallisation trials with purified enzyme will be carried out. Crystals of sufficient quality will be cryogenically preserved for X-ray diffraction experiments at the Diamond Synchrotron.

The *C. violaceum* omega transaminase should prove capable as a model for the structural determination and molecular modelling of the *S. avermitilis* omega transaminase for it has better sequence identity to the *S. avermitilis* and *P. aeruginosa*

omega transaminase's than they do for one another. The sequence identity between *C. violaceum* and *S. avermitilis* omega transaminases is 30 %, *C. violaceum* and *P. aeruginosa* omega transaminases 33 % while *S. avermitilis* and *P. aeruginosa* omega transaminase only have 23 % sequence identity (Fig. 1.12.)

The F89A mutant *C. violaceum* omega transaminase enzyme which had previously been crystallised by Julia Klose and a data set collected, will be refined in order to compare the structure with the wild type enzyme. Assays will be performed to determine whether mutating this residue allows the enzyme to use β -alanine.

Chapter 2 - Materials and Methods

All chemicals were obtained from Sigma-Aldrich unless specified otherwise. Where appropriate all microbiological equipment was sterilised before use by autoclaving at 121°C for 20 minutes. Solvents were all analytical grade and those used in HPLC assays were HPLC grade. Double distilled purite water (ddH₂O) was used wherever water was a necessary solvent unless specified otherwise.

2.1. Enzyme Purification

<u>2.1.1. Buffers</u>
Buffer A: Primary buffer
50 mM Tris-HCl pH 7.5/ 8.0/ 6.3
50 μM PLP

Buffer B: Nickel column buffer 50 mM Tris-HCl pH 7.5 1 M Imidazole 50 μM PLP

Buffer C: Gel filtration buffer 50 mM Tris-HCl pH 7.5 100 mM NaCl 50 μM PLP

Buffer D: Hydrophobic interaction chromatography buffer
50 mM Tris-HCl pH 7.0
1 M Ammonium sulphate
50 μM PLP

Buffer E: Ion exchange buffer 50 mM Tris-HCl pH 7.5/ 8.0/ 6.3 1 M NaCl 50 μM PLP

2.1.2. Bacteria Cultures and Storage

The cloned omega transaminase enzymes were provided by John Ward, UCL. *Escherichia coli* BL21 StarTM (DE3) pLysS cells were used containing the pET29a vector (Novagen, Nottingham UK) that possessed either the *S. avermitilis*, *P. putida* or *D. geothermalis* omega transaminase genes. The other species was *E. coli* BL21 Gold containing the vector pET-24a(+) (Novagen, Nottingham UK) that had the *P. aeruginosa* omega transaminase gene within it. All genes were His-tagged and the vector carried a gene for kanamycin resistance to allow selection of cells containing the vector. A T7 promoter lies upstream of the omega transaminase gene which requires the T7 polymerase whose expression is under the control of a *lac* operator that is repressed in the absence of lactose. The expression of the T7 polymerase and the omega transaminase gene is induced by addition of IPTG which is an analogue of lactose [33].

Glycerol stocks were made by mixing 50 µl of 50 % sterile glycerol (Fisher Scientific) and the same amount of bacteria culture and flash frozen in liquid nitrogen and then stored in a -80°C freezer. 100 µl of glycerol stock was added to 100 ml of lysogeny broth (LB) (25 % w/v) (MELFORD) with 100 µl of 50 mg/ml kanamycin under sterile conditions and incubated at 37°C at 200 rpm overnight. Then to each 1 litre flask, 20 ml of the overnight culture was added with 1 ml 50 mg/ml kanamycin and these were incubated under the same conditions until an optical density (OD) of 0.8 at 600 nm was reached at which point 1 ml IPTG (MELFORD) was added and then allowed to incubate for a further 4 hours. Cultures were then spun down at 12000 g for 30 min to obtain a cell paste. The supernatant was discarded and the paste was frozen and stored at -20°C. Overexpression of the respective omega transaminase was checked by running a SDS-PAGE gel and looking for a clear band around 50 kDa

2.1.3. Lysing Cell Cultures

For each 1 g of cell paste, 10 ml of Buffer A was used to resuspend the pellet. The *E.coli* cell paste over-expressing the *S. avermitilis* omega transaminase was much more viscous and difficult to resuspend so 7 μ l bezonase nuclease (\geq 250 units/ μ l) was added to digest the nucleic acid. Samples were then sonicated ten times for 15 seconds in ice with 45 seconds between sonications with a Soniprep 150 Sonicator

(Sanyo). The suspension was then centrifuged at 12000 g for 20 minutes and the supernatant poured off and the pellet disposed of.

2.1.4. SDS-PAGE gels

SDS-PAGE separates proteins based on their molecular weight. Gels were prepared in advance. The apparatus used was a Bio-Rad Mini-protein II vertical electrophoresis apparatus (Bio-Rad Laboratories Ltd). Gels were made between two glass plates 1 mM apart and water was added first to ensure no leaks and the plates dried again using filter paper. The separating gel was added until it was 2/3 full and allowed to polymerize at room temperature before adding the stacking gel and the comb to form the wells.

 Separating Gel:
 4.2 ml 30 % Acrylamide/bisacrylamide (29:1)

 2.5 ml 1.5 M Tris-HCl, pH 8.6

 1.0 ml 10 % SDS w/v

 2.3 ml ddH2O

 Stacking Gel:
 2.0 ml 30 % Acrylamide/bisacrylamide (29:1)

 2.5 ml 0.5 M Tris-HCl, pH 6.8

 1.0 ml 10 % SDS w/v

 4.5 ml ddH2O

10 μ l TEMED and 100 μ l APS (10 % w/v) were added to each of the gel solutions just before they were loaded into the apparatus. APS was made fresh each time and butan-2-ol was put on top of the separating gel to remove any bubbles and to stop it from drying out until it had polymerized enough for the stacking gel to be added.

10 µl of a sample and 10 µl of the loading buffer (100 mM Tris-HCl, pH 8.0, 2 % β mercaptoethanol v/v, 4 % SDS w/v, 0.2 % bromophenol blue w/v, 20 % glycerol v/v) were mixed together to denature the protein. The SDS binds to the protein unfolding it and adds a negative charge. The mercaptoethanol reduces any disulphide bonds. The sample was then put in a Heatblock for 5 minutes at 100°C. The samples were then added to the wells formed by the comb using a 50 µl Hamilton glass syringe. 5 µl of a molecular weight marker (ladder) was added to the first well (Biorad, 10-250 kDa). Gels were run for 50 minutes at 200 V in a running buffer containing 25 mM Tris, 192 mM glycine and 0.1 % SDS at pH 8.3. Staining was done by microwaving the gels in the stain (1 g Coomassie Brilliant Blue, 1 L methanol, 800 ml ddH₂O, 200 ml glacial acetic acid) for 5 minutes, then 15 minutes in water. An over-expression band corresponding to a molecular weight of 50 kDa, using the ladder as a control, verified the likelihood of the omega transaminase being present within the sample.

2.1.5. Purification of enzymes

An automated FPLC (GE Pharmacia AKTA Purifier) was used with the columns mentioned below in a cold room at 7°C.

2.1.6. Nickel Affinity Chromatography

A 10 ml Hiload column (Pharmacia) was equilibrated with three column volumes of Buffer A pH 7.5 with a flow rate of \leq 3 ml/min. Samples were applied to the column using a 10 ml or 50 ml Superloop and any unbound protein was washed out with three column volumes of Buffer A pH 7.5. Bound proteins were eluted with Buffer B up to a concentration of 100 %. Columns were washed afterwards with three column volumes of ethanol (Fisher Scientific). Absorbance was measured at 280 nm to determine which fractions contained the protein of interest which was then confirmed by running an SDS-PAGE gel.

2.1.7. Hydrophobic interaction chromatography

A 20 ml Phenyl Sepharose column (GE Healthcare) was equilibrated with three column volumes of Buffer D with a flow rate of \leq 3 ml/min. Samples in Buffer D were applied to the column using a 10 ml or 50 ml Superloop. Two column volumes of Buffer D were run through to wash out unbound protein and a linear gradient from Buffer D to Buffer A pH 7.5 over three column volumes was applied. Absorbance was measured at 280nm to determine which fractions contained proteins which were then tested by running an SDS-PAGE gel.

2.1.8. Ion exchange chromatography

A 12 ml anion exchange column (Fast Flow Q, GE Healthcare) was equilibrated with three column volumes of Buffer A at pH 7.5. This was one pH unit above the theoretical pI of the protein of interest as determined by ProtParam (Expasy tools, http://www.expasy.ch/tools/protparam.html). Samples from the Phenyl Sepharose

column dialysed against Buffer A pH 7.5 were loaded onto the column, three column volumes the same buffer was used to wash out unbound protein. A gradient from 0-1 M NaCl Buffer E pH 7.5 over five column volumes was used followed by 10 ml at 100 % Buffer E. The experiment was repeated at pH 8.0. Both failed to bind the protein of interest so the experiment was repeated using a 1 ml cation exchange column (Fast Flow S, GE Healthcare) with Buffers A and E at pH 6.3. Samples were injected using a 50 ml superloop, washed with 10 ml of Buffer A at pH 6.3 and eluted up to 100 % Buffer E pH 6.3 over a 20 ml gradient followed by 5 ml at 100 % Buffer E pH 6.3.

2.1.9. Gel Filtration Chromatography

Samples from the Nickel column were concentrated using a Vivaspin concentrator (Vivascience) with a 10 kDa cut-off to ≤ 2 ml. A GF200 column (Superdex 200 Hiload 16/60, GE Healthcare) was equilibrated with one column volume of Buffer C with a flow rate of ≤ 1 ml/min depending on the pressure. Samples were applied using a 2 ml injection loop and eluted with one column volume of Buffer C into 1.5 ml fractions. Absorbance was measured at 280 nm to determine the fractions that contained protein and the protein was determined by SDS-PAGE gels. Fractions with omega transaminase were then pooled together.

2.2. Crystallisation Trials

2.2.1. Protein Concentration Determination

A NanoDrop 2000c spectrophotometer (Thermo Scientific) was used to determine the concentration of protein samples. 2 μ l of Buffer C was added as a blank and was cleaned off with a medical wipe before adding 2 μ l of the protein sample. Each time it was ensured that there were no air bubbles in the droplet and the arm was lowered down properly before scanning. Samples were scanned at 280 nm and the concentration automatically calculated in mg/ml once provided with the extinction coefficient, from the ExPASy Proteomics Server by submitting the amino acid sequence of the protein in question, and the molecular weight of the protein. The sensor was cleaned before and after use with ethanol (Fisher Scientific) and then with distilled water using a medical wipe.

2.2.2. Crystal Screening

A concentration of 10 mg/ml is required for crystallisation so samples were spun down in a Vivaspin centrifugal concentrator (Vivascience) with a size cut-off at 10 kDa until the volume as determined by the equation $V_1 \ge C_2 \ge C_2$ is reached.

An Oryx 6 crystallisation robot (Douglas Research, UK) was used to set up microbatch crystallisation trials in a Hampton 96 well plate. The program used dispensed a 50:50 mix of protein: precipitation condition into each well to a volume of 1 μ l and covered each one in Al's oil (50 % silicon oil, 50 % paraffin oil). They were then incubated at 19°C and monitored under a light microscope for crystal growth. The screens used were Sigma 82009 and 70437 (Sigma Aldrich), MDL-01 and MDL-02, JCSG plus, MIDAS and Morpheus (Molecular Dimensions Limited).

2.2.3. Crystal Soaks

P. aeruginosa omega transaminase was the subject of these trials. Crystal soaks were done with crystals from a range of conditions (Table. 2.1.) which were then soaked for 15-30 seconds in an existing cryo-protectant with pyruvate added so it consisted of 50 μ M PLP, 100 mM NaCl, 50 mM Tris, 12 % w/v PEG 3350, 30 % v/v PEG 400 and 10 mM sodium pyruvate.

	Salt	Buffer	pН	Precipitant
Sigma 70437	None	None	-	10 % w/v PEG 1000 10 % w/v PEG 8000
Sigma 70437	0.2 M magnesium chloride	0.1 M Bis Tris	5.5	25 % w/v PEG 3350
JCSG plus	None	None	6.5	1.6 M tri-sodium citrate
JCSG plus	0.04 M potassium dihydrogen phosphate	None	-	16 % w/v PEG 8000/ 20 % v/v Glycerol
MD1-01	0.2 M magnesium chloride	0.1 M Tris	8.5	30 % w/v PEG 4000

Table. 2.1. List of conditions where *P. aeruginosa* omega transaminase crystals were formed which were used in crystal soaking experiments.

2.2.4. Co-crystallisation

Separate co-crystallisation trials were set up for *P. aeruginosa* omega transaminase with pyruvate and with gabaculine. Pyruvate co-crystallisation was repeated three times, each with a different concentration of sodium pyruvate. The protein concentration remained close to 10 mg/ml each time while sodium pyruvate was varied to 10 mM, 30 mM or 37.5 mM. Samples were mixed together and incubated at room temperature for 30 minutes before being used in crystal trials.

Gabaculine co-crystallisation was done by calculating the concentration of the protein in molarity (0.48 mM) and making a solution of gabaculine approximately double that of the protein since it is such a potent inhibitor, which in this instance was 1 mM. Equal amounts of gabaculine and protein solutions were mixed and incubated at room temperature for 30 minutes before being used in crystallisation trials.

2.2.5. Crystal Optimisation

X-step optimisation software (Douglas Instruments) was used to design crystallisation optimisation conditions. Two runs were set up both based on conditions that produced the best protein crystals from crystallisation trials. The first had the following condition and variables; Protein 3-6 mg/ml, Succinic acid 25-38 mM, PEG3350 5-13.625 % w/v, pH 5.41-6.14. The second was as follows; Protein 4.125-5 mg/ml, Tris 3.5-25 mM, PEG 8000 1.5-8.5 % w/v, pH 7.0-7.43.

2.2.6. Freezing Crystals

Crystal growth was monitored over a period of days or weeks and any crystals that were considered good enough to be used were frozen for future use. Crystals were mounted on nylon loops in the droplet under a microscope. Liquid nitrogen was used as the freezing agent and was also used to store the crystals in. Crystals were submerged for a few seconds in a cryo-protectant to prevent intrinsic ice formation which could damage the crystal or interfere with the X-ray diffraction patterns, where necessary. Cryo-protectants were initially individually tailored to the conditions of the well the crystals were grown in as well as the conditions they were eluted in during gel filtration. Where the precipitation conditions were similar in terms of the molecular weight of the PEG used, the cryo-protectants that were made earlier were used again for these. Table. 2.2. shows the list of cryo-protectants that were made, 50 μ M PLP was included in each.

Crystals obtained from the Morpheus screen were already cryo-protected and as such were frozen straight from the droplet. The same was done for conditions from other screens where the precipitant used was at a high enough concentration to act as a cryo-protectant itself.

pH	Salt	Buffer	Protectant 1	Protectant 2
7.0	-	50 mM Tris	12 % w/v PEG 3350	30 % v/v PEG 400
7.0	0.1 M NaCl	MgCl ₂	8 % w/v PEG 8000	30 % v/v PEG 400
8.5	0.2 M MgCl ₂	0.1 M Tris	22 % w/v PEG 4000	25 % v/v PEG 400
8.5	0.2 M trisodium citrate	0.1 M Tris	-	30 % v/v MPD

Table. 2.2. List of cryo-protectants made.

2.3. Assays

2.3.1. HPLC Assay

Two assays were carried out. The control assay to ensure enzymatic activity had not been lost contained the following; 25 mM MBA, 10 mM pyruvate, 100 mM HEPES pH 8.0 and 0.5 mM PLP. 0.5 mg of omega transaminase enzyme was added to the stock assay up to a total volume of 1 ml. The assay to detect β -alanine activity contained the following; 25 mM β -alanine , 10 mM acetophenone, 100 mM HEPES and 0.5 mM PLP. 0.5 mg of omega transaminase was added to a total volume of 1 ml as before and a repeat at a concentration of 2 mg of enzyme was also made. A crude lysate of the wild type *C. violaceum* omega transaminase was used as a control as a purified sample was not available at the time. Samples were shaken in an incubator at 800rpm and tested over time. 100 µl of the incubated samples was mixed with 100 µl acetonitrile, vortexed and then centrifuged at 13000 g for 1 minute. The supernatants were then transferred into HPLC vials with glass inserts in them so the sampler could

reach them. The absorbance was then measured at 250 nm over time for acetophenone production in an HPLC (Varian ProStar) [34].

The HPLC running program was as follows; 60 % H_2O and 40 % acetonitrile for 5 minutes. Then to 20 % H_2O and 80 % acetonitrile over 5 minutes and lasting for 30 seconds. 2 % H_2O and 98 % acetonitrile over 2 minutes lasting for 30 seconds before returning to 60 % H_2O and 40 % acetonitrile over 5 minutes.

Samples of purified F89A mutant omega transaminase were stored in a -80°C freezer prior to assays.

2.3.2. Tetrazolium Salt Based High-Throughput Assay

Colourimetric assays were carried out using 100 mM HEPES pH 8.0, 10 mM β alanine, 20 mM pyruvate, 5 µg/ml 1-Methoxy-5-Methylphenazinium Methyl Sulphate (mPMS), 40 µg/ml PLP, 0.2 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT), 0.5 mM NAD⁺ and 1.5 mg/3 mg of omega transaminase and alanine dehydrogenase whose amount has to be determined. The formation of the reduced form of the trizolium salt MTT, which possesses a dark blue colouration, was measured with mPMS used as a hydrogen carrier between NADH and MTT. Samples were analysed in a SpectraMax plate reader (Molecular Devices) and colour change monitored by detecting absorbance at 405 nm.

2.3.3. Amino Acid Oxidase/Horseradish Peroxidase Linked Assay

A stock assay was made to 10 ml containing 15 mM β -alanine, 0.2 mg/ml pyruvate, 100 mM HEPES pH 8.0, 4 mg/ml 2, 4, 6-tribromo-3-benzoic acid (TBHBA), 3 mg/ml 4-aminoantipyrine (4-AAP), 50 μ M PLP, 1 mg horseradish peroxidase and 8 units of L-amino acid oxidase. To 50 μ l of enzyme 100 μ l of stock assay was added in a 96 well microtiter plate (Greiner Bio-one) to a final enzyme concentration of 1 mg/ml. Positive and negative controls were made with 50 μ l Buffer A and 50 μ l 60 mM (final concentration 20 mM) DL-alanine. Absorbance was measured at 510 nm in a microplate reader (Tecan) [35].

2.3.4. Differential Scanning Fluorometry

A purified protein sample was diluted to 2.5 μ M concentration and to a volume of 4.2 ml with Buffer A pH 7.5. To this 4.2 μ l of 5000 x Sypro Orange was added. 16 μ l samples were mixed with 24 μ l of buffer listed in Table. 2.3. within a 96 well white quantitative PCR plate (Fisher Scientific) on ice with each condition repeated three times each for reliability. The plate was then sealed with optical sealing tape (Bio-Rad), gently shaken and then spun down in a centrifuge at 300 g for two minutes. Samples where then placed within a quantitative PCR machine (Stratagene Mx3005P) and heated to 25°C for 5 minutes. The temperature was then increased by 1°C each minute with a reading taken for every degree Celsius change in temperature. Data was then exported to Excel and analysed using Graphpad Prism to calculate the Tm values by using the Boltzmann equation (Fig. 2.1.) [31][32].

$$I = \begin{bmatrix} A + \frac{B - A}{1 + e^{(Tm - T)/C}} \end{bmatrix}$$

Fig. 2.1. Boltzmann equation.

Buffer	рН				
Tris	7	7.5	8		
Bis-Tris	6	6.5	7		
Bis-Tris Propane	6.5	7	7.5	8	
Bicine	7.6	8			
EPPS	7.5	8			
HEPES	7	7.5	8		
PIPES	6.6	7	7.5		
MOPS	6.5	7	7.5		
MES	6	6.5			
Trisodium citrate	6				
Potassium acetate	5.8				
Potassium phosphate	6	6.5	7	7.5	8

Table. 2.3. List of buffers and the range of pH's used in the Differential Scanning Fluorometry experiment.

2.4. Bioinformatics

2.4.1. Multiple Sequence Alignment

ClustalW from EBI Database was used to perform a multiple sequence alignment between *C. violaceum* (CV2025), *P. aeruginosa* (PA0132), *S. avermitilis* (SAV4551), *P. putida* (KT2440) and *D. geothermalis* (DSM11300) omega transaminases.

2.4.2. Homology Modelling

The software 'MOE' [36] was used to create a model of *S. avermitilis* omega transaminase based on the structure of the *C. violaceum* holoenzyme solved by Dr Christopher Sayer [24]. The 30 % sequence homology and the fact that it is part of the same sub-class of enzymes makes it a good template to base the structure on.

The known sequence for *S. avermitilis* omega transaminase was used as well as the PDB file for the structure of the *C. violaceum* holoenzyme. The sequences of the two were aligned and then superimposed to align them taking into account the predicted secondary structure. Amber 99 with a reaction field treatment of solvation electrostatics for force field predictions was used to create and destroy hydrogens and lone pairs as appropriate to assign partial charges and complete the outer valence electron shells.

2.4.3. X-ray Data Collection

Data was collected using the Diamond Synchrotron Light Source on beamline I03 by Dr Christopher Sayer with myself present. Crystals were kept in cryo-conditions within a stream of liquid nitrogen. Data was processed by the programs Xia2 [37] and MOSFLM [38]. Molecular replacement was performed by MOLREP [39].

2.4.4. Structural Refinement

The program COOT [40] was used to perform structural refinement between the structure and the electron density map, alterations were made manually based on both of the Fourier transformations 2Fo-Fc and Fo-Fc. Additionally it was used for the addition of water molecules to the model. REFMAC [41] was used between rounds of manual refinement with COOT to reduce the R factor and free R factor.

Chapter 3 - Results

3.1. Protein Purification

3.1.1. Successful Purification Experiments

SDS-PAGE gels were run after sonication to confirm the presence of the overexpressed omega transaminase enzyme. Strong bands within the appropriate size range were seen in all of the cloned omega transaminase enzymes from different species.

Purification of the cloned omega transaminases began with Nickel chromatography in an attempt to isolate the His tagged protein. SDS-PAGE were run to see if this was successful and as Fig. 3.1. and 3.4. show there were clear bands in wells three to five and three to six respectively that were approximately 50 kDa in size from one of the peaks. The second well contained a sample from the flow-through and showed that most of the other proteins were eluted in that one. However there were more bands in the other wells which means other proteins were still contaminating the sample. A second round of purification was performed by using gel filtration chromatography, traces are shown in Fig. 3.2. and Fig. 3.5. A clear and decisive peak was seen for each giving the probable location of the omega transaminase, which was confirmed by running further SDS-PAGE gels. Fig. 3.3. shows the results of gel filtration for the P. aeruginosa omega transaminase, 50 kDa bands were seen in wells three and four which were taken from either end of the large peak. The other wells contained sample from minor peaks seen in the trace, no other proteins were seen this time with the omega transaminase. The same was seen in Fig. 3.6. for S. avermitilis omega transaminase purification where the fifth well showed a significant amount of a second, smaller protein while the second, third and fourth wells possessed only a band around 50 kDa. Fig. 3.7. shows the results after gel filtration for P. putida omega transaminase where there was clear indication of successful purification as well.

Wild type *C. violaceum* omega transaminase was also purified to act as a control in the amino acid oxidase/horseradish peroxidise linked assay. The protocol for purification produced a pure sample after Nickel and gel filtration chromatographies as shown on the SDS-PAGE gel in Fig. 3.8.



Fig. 3.1. SDS-PAGE for *P. aeruginosa* omega transaminase after Nickel affinity chromatography with marker in well 1. Wells 3-5 indicating presence of omega transaminase, well 2 taken from flow-through.



Fig. 3.2. Trace from gel filtration chromatography for *P. aeruginosa* omega transaminase with green line indicating location of protein elution. Major peak seen between 60 and 70 ml. Minor peaks at 20 ml and 105 ml.



Lane 1 – Ladder Lane 2 – Before main peak Lane 3 – Peak sample Lane 4 – Peak sample Lane 5 – Subsequent peak sample Lane 6 – Subsequent Peak sample

Fig. 3.3. SDS-PAGE for *P. aeruginosa* omega transaminase after gel filtration chromatography. First well contained ladder, third and fourth wells show 50 kDa bands, other wells were taken from other peaks and show no other bands to those seen in the third and fourth.



Fig. 3.4. SDS-PAGE for *S. avermitilis* omega transaminase after Nickel affinity chromatography with marker in well 1. Wells 3-6 indicating presence of omega transaminase were contaminated with other proteins as can be seen from other bands, well 2 taken from the peak before imidazole was added.



Fig. 3.5. Trace from gel filtration chromatography for *S. avermitilis* omega transaminase with green line indicating location of protein elution. Major peak seen between 60 and 70 ml, minor peaks at 30 ml, 73 ml and 89 ml.



Fig. 3.6. SDS-PAGE for *S. avermitilis* omega transaminase after gel filtration chromatography. First well contained ladder, second, third and fourth wells show 50 kDa bands, other wells were taken from other peaks and show no bands similar to those seen in the third and fourth.



Fig. 3.7. SDS-PAGE for *P. putida* omega transaminase after gel filtration chromatography. First well contained ladder, the fifth, sixth, seventh, eighth and ninth wells show 50 kDa bands. Other wells were taken from other peaks and show no bands similar to those seen in the ones mentioned.



Fig. 3.8. SDS-PAGE for *C. violaceum* omega transaminase after gel filtration chromatography. First well contained ladder, the second, fifth, sixth and seventh wells contained small peaks on the trace. The third and fourth wells were from the main peak and contained 50 kDa bands.

3.1.2. Attempts at the Purification of D. geothermalis omega transaminase

The *D. geothermalis* omega transaminase required a more inventive approach to purify it as it failed to bind to the Nickel column despite being His-tagged. Hydrophobic interaction chromatography was used as an initial step to separate proteins based on their hydrophobic properties. Fig. 3.9. shows the trace from this column with two clear peaks as the concentration of ammonium sulphate decreased resulting in a less charged environment allowing elution of the protein which was confirmed by SDS-PAGE (Fig. 3.10.). The protein of interest was eluted in the second peak.

The next step was to perform ion exchange chromatography to separate proteins based on their ionic properties. A pH of 7.5 was initially used as it was close to 1 pH unit above the theoretical pI of the protein which was determined as 5.96 by ProtParam. Fig. 3.11. shows the trace from this and with the two peaks near the end of the gradient it was expected that one of these would contain the protein of interest. However after SDS-PAGE (Fig. 3.12.) it was shown that the protein had been eluted in the flow-through so the experiment was set up again with the buffers at a pH of 8 in case the estimated pI was too low. Again the same results were seen so a cation exchange column was set up and the pH of the buffer altered to 6.3 assuming the estimated pI was completely wrong. Again the protein of interest was eluted in the flow-through. However the protein that eluted in the flow-through of the FFQ column at pH 7.5 was relatively pure (Fig. 3.11.).



Fig. 3.9. Trace from hydrophobic interaction chromatography for *D. geothermalis* omega transaminase with green line indicating location of protein elution. The concentration of buffer D represented as the red trace. 2 peaks are seen as the concentration of buffer D decreases and the environment becomes less charged.



Fig. 3.10. SDS-PAGE for *D. geothermalis* omega transaminase after hydrophobic interaction chromatography. The first well contained the ladder, the second, third and fourth wells contained the flow-through. The fifth, sixth, seventh, eighth and ninth wells contained the first peak and the tenth well contained the second peak. Protein is 42 kDa so was located just below the 55 kDa marker in the tenth well as indicated by the arrow.

Lane 1 – Ladder Lane 2 – Flow through Lane 3 – Flow through Lane 4 – Flow through Lane 5 – First peak sample Lane 6 – First peak sample Lane 7 – First peak sample Lane 8 – First peak sample Lane 9 – First peak sample Lane 10 – Second peak



Fig. 3.11. Trace from ion exchange chromatography for *D. geothermalis* omega transaminase with green line indicating location of protein elution. The concentration of buffer E represented as the red trace. Two peaks are seen as the concentration of NaCl increased.



Fig. 3.12. SDS-PAGE for *D. geothermalis* omega transaminase after ion exchange chromatography using an anion exchange column. The first well contained the ladder, the second well contained a sample just before the flow-through, the third and fourth wells contained the flow-through, the fifth, sixth, seventh and eighth wells contained samples between the flow-through and the first peak. The eighth contained the first peak, the ninth the second peak and the tenth contained the end of the graph after the second peak. The protein is 42 kDa so was located close to the 40 kDa marker.

Lane 1 – Ladder Lane 2 – Before flow through Lane 3 – Flow through Lane 4 – Flow through Lane 5 – Before first peak Lane 6 – Before first peak Lane 7 – Before first peak Lane 8 – First peak Lane 9 – Second peak Lane 10 – After second peak

3.2. Protein Crystallisation and Structural Determination

All crystallisation trials were carried out by myself. The X-ray data collection and data analysis was carried out with Dr Michail Isupov. The refinement of the data from the *C. violaceum* F89A omega transaminase mutant was carried out by myself.

3.2.1. Crystallisation Trials

P. aeruginosa omega transaminase proved to be the easiest to crystallise with many protein crystals clearly visible in a variety of wells in each of the three screens used at the time; MDL, Sigma and JCSG plus. The most highly ordered crystals appeared to be very thin yet large, plate-like crystals (Fig. 3.13.). These were quite common and while fragile, proved to be capable of providing high resolution data of the holoenzyme at the Diamond Synchrotron. The data was collected at 1.7 Å resolution and was processed easily. This gave a good indication that this type of crystals which were seen later on, are possible of providing good data to work with.

While not as numerous as observed for the *P. aeruginosa* omega transaminase, *S. avermitilis* omega transaminase provided some crystals for testing. The best two conditions where there were a good number of good sized, disc-like crystals formed (Fig. 3.14.). The rest were mainly clusters of crystals which could not be separated. Both of these were from the JCSG-plus screen and are listed below, each had a cryo-protectant specifically designed for it;

B8: (0.2 M, MgCl₂, 0.1 M Tris, pH 7, 10 % w/v PEG 8000)

G7: (0.1 M succinic acid, pH 7, 15 % w/v PEG 3350).

While under the microscope these looked to be good, when tested with X-ray diffraction they were very disordered. The conditions for crystallisation have been optimised.

The crystals from B8 were put onto a loop and momentarily suspended in a cryoprotectant made of 50 μ M PLP, 100 mM NaCl, 200 mM MgCl₂, 8 % PEG 8000 and 30 % PEG 400, before being placed into a tube in liquid nitrogen. The same procedure was carried out with G7 with a different cryo-protectant of 50 μ M PLP, 100 mM NaCl, 50 mM Tris, 12 % PEG 3350 and 30 % PEG 400.



Fig. 3.13. *P. aeruginosa* omega transaminase protein crystals. The picture shows numerous plate-like crystals stacked on top of one another. The yellow colouration indicates presence of the PLP coenzyme which is yellow in its natural state. Crystallisation conditions used were 0.2 M magnesium chloride, 0.1 M Tris, 30 % w/v PEG 4000 at pH 8.5.



2.5mm

Fig. 3.14. JCSG plus crystal dish containing *S. avermitilis* omega transaminase protein crystals. Protein seems to form numerous small, disc shaped crystals. The yellow colouration indicates the presence of the PLP coenzyme which is yellow in its natural state. Conditions were 0.2 M MgCl₂, 0.1 M Tris, 10 % w/v PEG 8000 at pH 7.0. The largest and most ordered crystals were cryogenically frozen for future use.

3.2.2. Crystal Soaks

it.

Two of the *P. aeruginosa* omega transaminase crystals that were taken to the Synchrotron produced good data to a resolution of 2 Å, their growth conditions were 0.04 M potassium dihydrogen phosphate, 16 % w/v PEG 8000 and 20 % v/v Glycerol for the first. The second was 0.2 M magnesium chloride, 0.1 M Tris, 30 % w/v PEG 4000 at pH 8.5. There were problems concerning the space group of the second dataset which prevented molecular replacement. The first underwent molecular replacement successfully using MOLREP [39], when refined using REFMAC [41] an R factor of 0.187 and a free R factor of 0.242 was obtained. It was clearly apparent on inspection that there was nothing in the active site so no further work was done with

Space group	$P2_{1}2_{1}2_{1}$				
Unit cell parameters	109.65 Å	111.49	Å 135.03 Å	90 [°] 90	^o 90 ^o
Resolution	Overall	Low	High		
Low resolution (Å)	50.80	50.80	2.08		
High resolution (Å)	2.03	9.08	2.03		
Rmerge	0.090	0.040	0.642		
I/sigma	9.9	21.6	2.7		
Completeness (%)	99.5	98.1	99.9		
Multiplicity	5.3	4.9	5.5		

Fig. 3.15. Statistics from X-ray data collected at the Diamond Synchrotron for the *P. aeruginosa* omega transaminase and pyruvate crystal soak whose growth conditions were 0.04 M potassium dihydrogen phosphate, 16 % w/v PEG 8000 and 20 % v/v Glycerol.

3.2.3. Co-crystallisation trials

Purified *P. aeruginosa* omega transaminase was concentrated to higher concentrations to compensate for the dilution involved. Initially gabaculine was added to a final concentration of 20 mM as was previously described [24]. However for this protein there was an obvious and immediate denaturation upon adding the gabaculine. Since it was such a potent inhibitor the concentration was reduced to approximately double that of the protein to a concentration of 1 mM which prevented this from happening. Five different screens were set up, of these MDL and Sigma did not provide any viable crystals. The screens and conditions that provided crystals that were good enough to freeze for future data collection are listed below in Table. 3.1. Only four of the crystals were scanned due to limited time during the last Synchrotron trip. Three of these were from a single well, its conditions were 60 mM MgCl₂; CaCl₂, 0.1 M

Tris; Bicine, 30 % PEGMME 550; PEG 20000 at pH 8.5. None of the crystals from this well produced good diffraction. The fourth well did provide data that could be used. Its conditions were 90 mM NaF; NaBr; NaI, 0.1 M Tris; Bicine, 30 % PEGMME 550; PEG 20000 at pH 6.5 and is shown in Fig. 3.16. Data was collected to 2.2 Å resolution and had a space group C222 however it was marred by ice rings and the data collected was very weak and could not be processed (Fig. 3.19.).

	Salt	Buffer	pН	Precipitant
Morpheus	60 mM MgCl2;	Tris; Bicine	8.5	30 % PEGMME
	CaCl2	0.1 M		550; PEG 20000
	90 mM NaF;	Tris; Bicine	6.5	30 % PEGMME
	NaBr; NaI	0.1 M		550; PEG 20000
	90 mM NaN03;	Tris; Bicine	6.5	30 % PEGMME
	Na2HPO4;	0.1 M		550; PEG 20000
	(NH4)2SO4			
MIDAS	10 % v/v	0.1 M MES-	6.0	30 % w/v
	ethanol	NaOH		polyacrylate 5100
				sodium salt
JCSG plus	0.2 M LiSO4	0.1 M sodium	4.5	50 % v/v PEG
		acetate		400
	None	None	6.5	1.6 M tri-sodium
				citrate
	0.2 M LiSO4	0.1 M sodium		30 % w/v PEG
		acetate		8000
	None	0.1 M Tris	8.0	20 % v/v MPD
	None	0.1 M Na	5.0	3.2 M ammonium
		Citrate		sulphate

Table 3.1. List of screens which produced crystals that were frozen from *P. aeruginosa* cocrystallisation experiments with gabaculine.

P. aeruginosa omega transaminase was co-crystallised with pyruvate and screens were made with the three different concentrations at 10 mM, 30 mM and 37.5 mM sodium pyruvate after incubation. The screens and conditions that provided crystals that were good enough to freeze down are listed below in Table. 3.2. Only one was scanned due to the limited time during the last Synchrotron trip. Its conditions were 0.1 M sodium citrate, 20 % w/v PEG 3000 at pH 5.5. Data was collected to 1.8 Å resolution and was assigned the same space grouping as the gabaculine co-crystallisation with a space grouping $P2_12_12_1$ with four subunits (Fig. 3.21.).

The data were successfully processed by Xia2 3dr [37] during data collection, however there were issues when it came to molecular replacement with MOLREP [39] as after refinement, converged with a free R of 0.41. There were breaks in the main chain electron density that indicated that the space group assignment might be incorrect. There were only two strong rotational peaks suggesting two subunits in the asymmetric unit, or possibly four subunits with similar orientation. Molecular replacement using MOLREP checked all possible space groups and suggested the best solution was a P2₁2 2 with a correlation of 0.33. However this solution had only two monomers per asymmetric unit and could not be refined to a free R better than 0.47. In the end the data was submitted to York Structural Biology Laboratory program server to run Zanuda [42] which assigned the correct space grouping of P2₁2₁2 where the solution could be refined to a free R below 0.25. Upon examination of the active site it was clear that there was no pyruvate within it. (Work done under Dr Michail Isupov).

	Salt	Buffer	pН	Precipitant
JCSG	0.2 M NaCl	0.1 M sodium cacodylate	6.5	2.0 M ammonium sulphate
	0.2 M ammonium chloride	None	-	20 % w/v PEG 3350
	None	0.1 M sodium acetate	4.6	2.0 M ammonium sulphate
	None	0.1 M sodium citrate	5.5	20 % w/v PEG 3000
	None	0.1 M imidazole	8.0	10 % w/v PEG 8000
MDL	0.2 M potassium sodium tartrate	0.1 M Na citrate	5.6	2.0 M ammonium sulphate

Table 3.2. List of screens which produced crystals that were frozen from *P. aeruginosa* cocrystallisation experiments with pyruvate.



2.5mm

Fig. 3.16. Morpheus crystal dish containing *P. aeruginosa* omega transaminase protein crystals co-crystallised with gabaculine. A single diamond shaped crystal was seen. The condition was 90 mM NaF; NaBr; NaI, 100 mM Tris; Bicine, 30 % PEGMME 550; PEG 20K at pH 6.5.



2.5mm

Fig. 3.17. JCSG plus crystal dish containing *P. aeruginosa* omega transaminase protein crystals co-crystallised with pyruvate. Numerous thin plate-like crystals were seen. The condition was 0.1 M sodium citrate, 20 % w/v PEG 3000 at pH 5.5.

C222			
122.50 Å	À 192.0	Å 77.30 Å	90° 90° 90°
Overall	Low	High	
29.51	29.51	2.32	
2.26	10.10	2.26	
0.156	0.060	0.702	
6.70	16.00	1.20	
97.8	95.7	80.1	
5.2	4.8	4.5	
	C222 122.50 Å Overall 29.51 2.26 0.156 6.70 97.8 5.2	C222 122.50 Å 192.0 Overall Low 29.51 29.51 2.26 10.10 0.156 0.060 6.70 16.00 97.8 95.7 5.2 4.8	C222 122.50 Å 192.0 Å 77.30 Å Overall Low High 29.51 29.51 2.32 2.26 10.10 2.26 0.156 0.060 0.702 6.70 16.00 1.20 97.8 95.7 80.1 5.2 4.8 4.5

Fig. 3.18. Statistics from X-ray diffraction at Diamond Synchrotron for gabaculine cocrystallisation whose growth conditions were 90 mM NaF; NaBr; NaI, 0.1 M Tris; Bicine, 30 % PEGMME 550; PEG 20000 at pH 6.5.



Fig. 3.19. X-ray diffraction pattern for *P. aeruginosa* omega transaminase protein crystals cocrystallised with gabaculine from conditions 90 mM NaF; NaBr; NaI, 100 mM Tris; Bicine, 30 % PEGMME 550; PEG 20000 at pH 6.5. Despite being cryo-protected, ice rings were seen. Diffraction was to 2.2 Å resolution.

Space group	P21212			
Unit cell parameters	111.96 Å	192.20	Å 76.70 Å	90° 90° 90°
Resolution	Overall	Low	High	
Low resolution (Å)	30.05	30.05	1.84	
High resolution (Å)	1.79	8.00	1.79	
Rmerge	0.137	0.053	0.716	
I/sigma	11.90	27.90	3.10	
Completeness (%)	99.7	97.9	96.2	
Multiplicity	13.1	11.7	12.1	

Fig. 3.20. Statistics from X-ray diffraction at the Diamond Synchrotron for pyruvate cocrystallisation.



Fig. 3.21. X-ray diffraction pattern for *P. aeruginosa* omega transaminase protein crystals cocrystallised with pyruvate from conditions 0.1 M sodium citrate, 20 % w/v PEG 3000 at pH 5.5. There were no ice rings and data was collected to 1.86 Å resolution.

3.3. Structural Analysis

3.3.1. Multiple Sequence Alignment

A multiple sequence alignment was done to compare the differences in the primary sequence of the five omega transaminases. Based on structural studies the residue phenylalanine at position 89 was the target for mutagenesis in *C. violaceum* omega transaminase to allow it to utilise β -alanine as a substrate. This was still conserved in the sequence alignment (Fig. 3.22.) of the *S. avermitilis* and the *P. aeruginosa* omega transaminase which both have activity to β -alanine. However using the known structures of the *C. violaceum* and *P. aeruginosa* omega transaminases [24] F89 was displaced by one residue and did not superimpose between these two enzymes. Therefore it was difficult to infer the importance of F89 from the sequence alignment alone. The *S. avermitilis* omega transaminase was missing one of the tryptophan residues, at position 60, that is proposed to restrict the active site of other omega transaminases [25].

All the omega transaminases possessed the K288 (*C. violaceum* numbering) as was expected for they all bind PLP. D259 (*C. violaceum* numbering), responsible for hydrogen bonding with PLP, was present in all of the enzymes except for the *D. geothermalis* omega transaminase where it appeared to have been replaced by a proline residue.

CV2025. KT2440. PA0132. SAV4551. DSM11300.	MQKQRTTSQWRELDAAHHLHPFTDTASLNQAGARVMTRGEGVYLWDSEGNKIIDG MATPSKAFAIAHDPLVEADKAHYMHGYHVFDEHREQGALNIVAGEGAYIRDTHGNRFLDA MNQPLNVAPPVSSELNLRAHWMPFSANRNFQKD-PRIIVAAEGSWLTDDKGRKVYDS MTPQPNPQVGAAVKAADRAHVFHSWSAQELIDPLAVAGAEGSYFWDYDGRRYLDF VEVEPRVLLEL	55 60 56 55 33
CV2025. KT2440. PA0132. SAV4551. DSM11300.	MAGLWCVNVGYGRKDFAEAARRQMEELPFYNTFFKTTHPAVVELSSLLAEVTPAGFDRVF VGGMWCTNIGLGREEMALAIVDQVRQLAYSNPFSDMANDVAIELCQKLAQLAPGDLNHVF LSGLWTCGAGHSRKEIQEAVARQLGTLDYS-PGFQYGHPLSFQLAEKIAGLLPGELNHVF TSGLVFTNIGYQHPKVVAAIQEQAASLTTFAPAFAVEARSEAARLIAERTPGDLDKIF AQPQIHHRTPQGIERLMEARAKLTRLLGDPYDAVITTSSGTGAFEGALISLTP * * * * * * *	115 120 115 113 86
CV2025. KT2440. PA0132. SAV4551. DSM11300.	YTNSGSESVDTMIRMVRRYWDVQGKPEKKTLIGRWNGYHGSTIGGASLG-GMKYMHEQGD LTTGGSTAVDTAYRLIQYYQNCRGKPHKKHIIARYNAYHGSTTLTMSIGNKAADRVPEFD FTGSGSECADTSIKMARAYWRLKGQPQKTKLIGRARGYHGVNVAGTSLG-GIGGNRKMFG FTNGGADAIEHAVRMARIHTGRPKVLSAYRSYHGGTQQAVNIT-GDPRRWASDS EGGRVVNAQAGKFSERWGDMSERLGYETVRVARPWGDLLDPEEVA .* ::. *	174 180 174 166 131
CV2025. KT2440. PA0132. SAV4551. DSM11300.	LPIPGMAHIEQPWWYKHGKDMTPDEFGVVAARWLEEKILEIGADKVAAFVGEPIQGAGGV YHHDLIHHVSNPNPYRAPDDMDEAEFLDFLVAEFEDKILSLGADNVAAFFAEPIMGSGGV QLMDVDHLPHTLQPGMAFTRGMAQTGGVELANELLKLIELHDASNIAAVIVEPMSGSAGV ASAGVVHFWAPYLYRSRFYAETEQQECERALEHLETTIAFEGPGTIAAIVLETVPGTAGI DAARNAHTLLITHSETSTGALHDLAAIAQAAKAQNPDLVIIADCITSYGVAELRPAAWGV : : * .: *:	234 240 234 226 191
CV2025. KT2440. PA0132. SAV4551. DSM11300.	IVPPATYWPEIERICRKYDVLLVADEVICGFGRTGEWFG-HQHFGFQPDLFTAAKGLSSG IIPPEGYFQRMWQLCQTYDILFVADEVVTSFGRLGTFFASEELFGVTPDIITTAKGLTSA LVPPVGYLQRLREICDQHNILLIFDEVITAFGRLGTYSG-AEYFGVTPDLMNVAKQVTNG MVPPPGYLAGVRELCDKYGIVFVLDEVMAGFGRTGEWFA-ADLFDVTPDLMTFAKGVNSG DVIVSGSQKGAATPPG-LGFVLLSPEVQERMIPTPQRGFYLDLTRELKGQKAG :	293 300 293 285 243
CV2025. KT2440. PA0132. SAV4551. DSM11300.	YLPIGAVFVGKRVAEGLIAGGDFNHGFTYSGHPVCAAVAHANVAALRDEGIVQR YLPLGACIFSERIWQVIAEPGKGRCFTHGFTYSGHPVCCTAALKNIEIIEREQLLDH AVPMGAVIASSEIYDTFMNQALPEHAVEFSHGYTYSAHPVACAAGLAALDILARDNLVQQ YVPLGGVAISGKIAETFGKRAYPGGLTYSGHPLACAAAVATINVMAEEGVVEN NTPQTPAINLISALSLALDRLLAVPLEVLWAEKRRQANALIAA * :.: : : : : : : : : : : : : : : : : :	347 357 353 338 286
CV2025. KT2440. PA0132. SAV4551. DSM11300.	VKDDIGPYMQKRWRETFSRFEHVDDVRGVGMVQAFTLVKNKAKRELFPDFGEIGTL VN-DVGSYLEQRL-QSLRDLPLVGDVRCMKLMACVEFVANKASKALFADEVNIGER SA-ELAPHFEKGL-HGLQGAKNVIDIRNCGLAGAIQIAPRDGDPTVRPFEAGM AANLGARVIEPGLRELAERHPSVGEVRGVGMFWALELVKDRETREPLVPYNAAGEANAPM GEALGAPAWAARTSPAVAVLKPPAPLTGRQVAARLAAMGQRALPGQAPYEDAVFRVS . : : .	403 411 404 398 343
CV2025. KT2440. PA0132. SAV4551. DSM11300.	CRDIFFRNNLIMRACGDHIVSAPPLVMTRAEVDEMLAVAERCLEEFEQTLKARGLA IHSKAQEKGLLVRPIMHLNVMSPPLIITHAQVDEIVETLRQCIIETARELTALGLYQG KLWQQGFYVRFGGDTLQFGPTFNARPEELDRLFDAVGEALNGIA AAFGAAAKANGLWPFINMNRTHVVPPCNVTEAEAKEGLAALDAALSVADEYTV TLGYADRYDALGIAGMLEDAFASLGVPFERGAAVQAAWRVLGEERREMAGCEAR 	459 469 448 451 397
CV2025. KT2440. PA0132. SAV4551. DSM11300	- R 470 - -	

Fig 3.22. Sequence alignment for *C. violaceum* (CV2025), *P. aeruginosa* (PA0132), *S. avermitilis* (SAV4551), *P. putida* (KT2440) and *D. geothermalis* (DSM11300) omega transaminases using ClustalW from EBI Database. "*" means that the residues in that column are identical for all the sequences in the alignment. ":" means that there are conserved substitutions based on the properties of the amino acids. "." means that semi-conserved substitutions are observed. Conserved residues as discussed in 3.3.1 are highlighted in red.

3.3.2. Homology Modelling Analysis

Through homology modelling, insight into the structure of the S. avermitilis omega transaminase can be determined. The template used for the homology modelling was the C. violaceum omega transaminase holoenzyme. The model was made using MOE (Molecular Operating Environment) with the amber99 force field. The final structure that was made had an RMSD of 0.884 Å indicating there was a strong similarity between the homology model and the template structure. Fig. 3.23. shows the two structures superimposed upon one another. Analysis of the active site (Fig. 3.24.) showed that K288 and F89 were in the same locations as those on the C. violaceum omega transaminase. The major difference that was observed was the absence of the R416 residue. Examination of the strand region it should be located on revealed that there was no carboxyl binding region present on it. The complete absence of this key residue seems unlikely as it is required for substrate binding. The most probable reason for this is that the carboxyl binding group is present on a different substructure within the active site. Close to where the R416 should be located was another arginine residue on a nearby loop region, R405, which seemed to be the most probable candidate since there was 6 Å distance between the closest nitrogen groups (Fig. 3.24.).



Fig. 3.23. *S. avermitilis* omega transaminase homology model, shown in red. The template, *C. violaceum* omega transaminase, is superimposed upon it and displayed in green. Both chains show high similarity to one another with a root-mean-square deviation of 0.884 Å. Image made using PyMOL [26].



Fig. 3.24. Homology modelling using MOE to show difference in key arginine residues using PyMOL [26]. PLP is displayed in yellow and the F89 is displayed in black. The *C. violaceum* omega transaminase chain is shown as green with R416 displayed in blue. The *S. avermitilis* omega transaminase chain and R405 are shown in red. The distance between the closest nitrogen groups of R405 and R416 was 6 Å.



Fig. 3.25. Ramachandran plot of the *S. avermitilis* omega transaminase homology model using the software Procheck [43].

3.3.3. Structural Refinement of Mutant C. violaceum omega transaminase

An R factor of 0.2014 and a free R factor of 0.2376 were achieved through structural refinement with COOT [40] and REFMAC [41]. This subsequently proved the mutant to have the same overall folding pattern as the native enzyme as is displayed in Fig. 3.26. which displays the F89A mutant in red superimposed upon the native *C. violaceum* omega transaminase. The key amino acid residues remained conserved within the active site with very little difference in their positions. The mutation of F89 to A89 can be seen in Fig. 3.27. The mutation of F89 can clearly be seen to be successful and loss of the phenylalanine side chain clearly provided more room within the active site. It resulted in a shift in the C α backbone as is shown in Fig. 3.27. which

allowed even more room for substrate binding within the active site. The R416 side chain was seen to move closer to the location where the phenylalanine side chain was once located which indicated it now possessed greater flexibility since it can now inhabit space it was previously unable to.

Using COOT [40] β -alanine was placed within the active site to determine the viability of it binding to the active site after mutation of F89 (Fig. 3.29.). Although there was now greater flexibility of R416 to bind and assume an orientation for catalytic activity with the substrate, there seemed to be a phenylalanine at position 88 which could prevent it binding. The side chain appeared to prevent the β -alanine from attaining a position where it could bind to the PLP and R416. Testing of the F89A mutant in an activity assay would determine if the single amino acid residue change was adequate to allow β -alanine to be utilised as a substrate.



Fig. 3.26. F89A mutant, shown in red, superimposed by the native *C. violaceum* omega transaminase, shown in green using PyMOL [26]. The overall fold of the protein remained mainly unaltered by the mutation.



Fig. 3.27. The 2Fo-Fc electron density map is shown in blue contoured at 1 sigma level with the model of the F89A mutant protein fitted in (carbons are shown in green). Also shown is the superimposed refined structure of the native protein (carbons are shown in yellow). Mutation of F89 to A89 caused a shift in the loop region it is on away from the active site and provided more room for substrate binding. The R416 was seen to move closer to where the phenylalanine side chain used to be, indicating there to be greater flexibility. Image made using COOT [40].



Fig. 3.28. PyMOL image displaying the shift in the loop region and the altered position of the R416 in the F89A mutant, shown in red, compared to the holoenzyme structure, shown in green [26].



Fig. 3.29. β -alanine placed within the active site of F89A mutant using COOT [40] and image made with PyMOL [26]. The purple chain represents the native enzyme, the green chain represents F89A mutant. F88 could prevent binding of the substrate due to steric hindrance.

3.4. Activity Assays

HPLC and tetrazolium salt based assays were carried out at UCL with Professor John Ward and Dr Nina Richter.

Results from HPLC assays are shown in Table. 3.3. with the omega transaminase CV2025 from *C. violaceum* in the form of a crude lysate as control. MBA assays were performed to confirm that the omega transaminase had retained its activity. An increase in acetophenone was observed which indicated that the F89A mutant omega transaminase still possessed enzymatic activity. Samples at 0.5 mg of enzyme tested for activity towards β -alanine showed no decrease in acetophenone concentration compared to the blank. A second sample at a higher concentration of 2 mg of enzyme was made, however this failed to provide any decrease in acetophenone concentrations compared to the blank either.

As the equilibrium lied in favour of acetophenone production, the HPLC assay was attempting to drive the reaction in an unfavourable direction with none of the products removed to help maintain conversion. This might have given a negative result if the F89A omega transaminase had very low activity towards β -alanine. So a colourimetric assay was performed by utilising the conversion of NAD⁺ to NADH by an Alanine Dehydrogenase as it converted alanine to pyruvate. This removed one of

the products and regenerated one of the substrates to ensure the reaction was not halted by reaching equilibrium. Analysis of the wells showed no colour change had occurred within the wells containing the F89A mutant. The only colour change observed was in the *C. violaceum* omega transaminase well, however since this was a crude lysate there are most likely many NAD⁺ \rightarrow NADH systems within the sample.

A second colourimetric assay was performed as the previous one had was still under development at UCL. This one was based on an L-amino acid oxidase converting the alanine back into pyruvate thus regenerating the amine acceptor, producing hydrogen peroxide. This product is utilised by the horseradish peroxidase to produce a quinone-imine dye from TBHBA and 4-AAP. After incubating overnight at room temperature a subtle colour change was visible as is seen in Fig. 3.30. with three repeats of both the wild type CV2025 and the F89A mutant. The bottom two wells contained the positive and negative controls. The negative control had an absorbance of 0.7289. The CV2025 wild type wells had mean absorbances of 0.7337, 0.7379 and 0.7300, none of which were significantly higher than the negative control. The F89A mutant showed a slightly different colour than that seen in both the wild type and negative control. Its absorbance's were 0.7689, 0.7645 and 0.7862. This small but consistent increase in absorption indicated there to be a small increase in alanine being produced by the F89A mutant compared to the blank and wild type based on the visual colour change.

Assay	Sample	Protein	Incubation	Area of	Acetophenone
		Concentration/mg	Time/hours	Peak	conetration/mM
MBA	Blank	0	0	9699	0.006
β-alanine	Blank	0	0	15881114	9.41
MBA	Blank	0	0.5	27476	0.02
MBA	F89A	0.5	0.5	7307780	4.33
MBA	CV2025	0.5	0.5	624539	0.370033285
β-alanine	Blank	0	0.5	15540892	9.21
β-alanine	F89A	0.5	0.5	16188485	9.59
β-alanine	CV2025	0.5	0.5	16648912	9.864318482
β-alanine	Blank	0	4	15671678	9.29
β-alanine	F89A	0.5	4	15792490	9.36
β-alanine	CV2025	0.5	4	15502498	9.185079334
β-alanine	Blank	0	21	15027740	8.90
β-alanine	F89A	0.5	21	14632531	8.67
β-alanine	CV2025	0.5	21	14454644	8.564236027
β-alanine	Blank	0	0	16323606	9.67
β-alanine	Blank	0	1	15807653	9.37
β-alanine	F89A	2	1	15742973	9.33
β-alanine	CV2025	2	1	15814363	9.369856314
β-alanine	Blank	0	17	13288295	7.87
β-alanine	F89A	2	17	13843313	8.20
β-alanine	CV2025	2	17	13769354	8.158208365

Table. 3.3. Results from the HPLC assays.



Fig. 3.30. Image of microtiter plate from amino acid oxidase/horseradish peroxidase linked assay. Bottom two wells contained the negative and positive controls, the latter of which clearly possessed a significant difference in colour. F89A mutant with the naked eye alone appeared to have a slightly different colour.

3.5. Differential Scanning Fluorometry

Fig. 3.31. shows the Tm values for each well of the differential scanning fluorometry trials with *S. avermitilis* omega transaminase as determined by the Boltzmann equation within Prism (GraphPad). Wells G7, G8 and G9 which contained potassium acetate pH 5.8 were empty due to anomalous Tm values of 140.6°C, 95.51°C and 212.8°C respectively. When examining the graphs themselves the curves appeared to be similar to the others with no obvious errors and no reason for these values could be found when examining the data put into Prism. This would need to be repeated to determine the Tm for this buffer.

Tris at pH 7.5 was used as a control (Fig 3.32.). The graph showed two peaks, each one indicating the unfolding of a different subunit of the dimer. The average Tm value was 55°C which was close to the other two Tris buffers, pH 7 had an average Tm of 56.6°C and pH 8 had a Tm of 58.8°C. These were considerably lower than the other average Tm values for the rest of the buffers tested where the majority ranged between 68°C and 71°C. Apart from the Tris buffers, Bis-Tris propane at pH 8 had the lowest average Tm value at 66°C, but even this was much higher than the three Tris buffers tested. The highest average Tm was for MES at pH 6 whose value was calculated to be 75.8°C. The graph in Fig. 3.33. showed a significant decrease in the amount of unfolding of the first subunit as the first peak in comparison with the first was much smaller signifying an increase in the thermodynamic stability of the protein. This average only took into account two of the datasets as one was 71.1°C which was 4.28°C lower than the second lowest value and possibly an outlier due to the difference from the other two. The second and third highest after this was potassium phosphate pH 6.5 with an average Tm of 72.5°C and pH 6 with an average Tm of 72.4°C.

Unfortunately these results are not reliable as the Boltzmann equation used is valid only for single transitions. A double transition is seen here from the unfolding of each subunit of the dimer. As this is the case the analysis method used is limited and other forms of analysis must be considered in the future such as calculating the first or second derivatives.



Fig. 3.31. Bar chart showing different Tm values for each well as calculated by Prism. Wells G7-G9 are not shown due to anomalous results. Wells listings are on the Y axis, temperature is on the X axis in degrees Centigrade.





Fig. 3.32. Graph showing change in fluorescence which corresponds to the unfolding of the protein for the control, Tris at pH 7.5. Dark blue data considered unreliable.



Fig. 3.33. Graph showing change in fluorescence which corresponds to the unfolding of the protein for MES at pH 6. Dark blue data considered unreliable.

Chapter 4 - Discussion

The inability of the *D. geothermalis* omega transaminase to bind to Nickel columns was most likely the result of the histidine tag becoming buried within the protein itself. Given time an alternate means of purification might be found, for the only thing that stopped attempts at purification was lack of time. The presence of the His-tag would have to be confirmed by means of Nickel chromatography with urea or guanidinium chloride to denature the protein and see if binding occurs. This would confirm whether the His tag has been lost or buried. If the latter were to be proved true then a new construct would have to be made with the His-tag located at the C-terminal of the protein.

The processing of X-ray data obtained from the *P. aeruginosa* omega transaminase proved to be a challenge due to incorrect assignment of the space group. This was probably due to a pseudo translation, tiny rotational or conformational differences causing modulation of intensities and leading to the doubling in size of the unit cell. Also the assignment of a space group was made difficult by the protein possessing a two fold proper axis that is not crystallographic, parallel to a screw axis that is crystallographic. The fact that the program Zanuda gave a space group of $P2_12_12$ confirms this, as here the two fold proper axis is considered crystallographic so a greater value in correlation calculations is obtained. (All work done under Dr Michail Isupov)

The failure to get the structure of *P. aeruginosa* omega transaminase with pyruvate within the active site was disappointing. Although acceptor substrate-pyridoxal phosphate intermediates were predicted to exist in the 1960's, no one so far has managed to observe them with one exception. The concentration of sodium pyruvate used was very high at 30 mM and near the limit of what could be used so using a higher concentration of pyruvate is not an option. This along with the data from the crystal soaks indicates that trapping a stable complex of PLP with pyruvate in omega transaminases is difficult. The data that was obtained by Dr Christopher Sayer [24] with *C. violaceum* transaminase where pyruvate is seen within the active site appears to be unique and difficult to repeat with others omega transaminases.

It is unknown whether gabaculine successfully co-crystallised with *P. aeruginosa* omega transaminase due to the poor quality of the data retrieved. Given what is known about the binding of gabaculine in previous work with the *C. violaceum* omega transaminase, it is highly likely that there was binding to the *P. aeruginosa* omega transaminase since gabaculine is such a highly potent inhibitor. It was concluded that the quality of the crystal used in the data collection prevented us from seeing this. Improved crystals were taken to the Diamond Synchrotron in March.

During the work with *P. aeruginosa* omega transaminase several new crystal forms were observed. The crystals were thin which made it hard to ensure that they were hit with the X-ray beam throughout all 360° of data collected, however they proved to provide high resolution data.

The failure of *P. putida* omega transaminase to crystallise properly is not uncommon in any crystallisation trial. It might be that one of the new screens that became available near the end of the experiment, the Morpheus and MIDAS, would successfully crystallise the protein.

The mutation of F89 to A89 in the C. violaceum omega transaminase was shown clearly in the crystal structure after structural refinement. The overall structure was conserved in the mutant protein. The objective of the mutation was to create more room in the active site and greater flexibility of the carboxyl binding group R416, both of which were observed. The backbone chain on which the A89 was present showed movement away from the active site which, along with the loss of the phenylalanine side chain, would allow more space for substrates to bind. Also the R416 showed movement towards the location where the F89 side chain had been, indicating the arginine side chain could now occupy a conformation which previously would have previously been hindered. However from looking at the active site with βalanine placed within it suggests the possibility that this mutation alone might be inadequate for activity with this substrate. Testing with both HPLC and tetrazolium salt based high-throughput assays both showed there was no detectable activity towards β -alanine. However the amino acid oxidase/horseradish peroxidase linked assay showed there to be some small difference when left for a long period of time which could indicate that there is a small amount of activity towards β -alanine due to the F89A mutation. This might have been previously undetected as the HPLC assay was going against the equilibrium and the tetrazolium salt based high-throughput assay was still being established at the time and had not been given as long to run. While the activity is not as high as was expected initially, there does appear to be limited success in endowing the ability of the mutant to utilise β -alanine as a substrate.

So although the mutation was based upon the *P. aeruginosa* omega transaminase which utilises β -alanine as a substrate, it is clear that this is insufficient to provide any significant activity and further comparison between the two structures is required. A mutation of the F88 might go some way to allow binding of β -alanine as docking experiments indicate it might cause steric hindrance towards binding larger substrates such as β -alanine.

The differential scanning fluorometry experiment with *S. avermitilis* omega transaminase clearly showed that the standard Tris buffer that had been used was by far the worst possible buffer to use when compared to those that were screened. It is unlikely that this was due to the pH changes that Tris undergoes when the temperature is altered as the pH 8 Tris buffer would have had a Tm over 10°C higher than the pH 7.5. This is concluded as a temperature change from 25°C to 37°C results in a change in pH of approximately 0.3 for each of the pH's meaning an increase of 0.5 pH units should result in a considerably higher Tm if denaturing is due solely to an increase in acidity. With a difference in Tm of 20°C between Tris at pH 7.5 and MES at pH 6, it is possible that ordered crystals might be obtained should the buffer be changed to this. This is due to the large increase in thermodynamic stability and under these conditions which should allow more ordered crystals to be grown which diffract to high resolution.

Chapter 5 - Conclusion

5.1. Summary of Work

Crystallising *P. aeruginosa* omega transaminase with pyruvate does not appear possible for both crystal soaking and co-crystallisation with pyruvate at a high concentration failed. It would seem unlikely therefore to be able to repeat the experiment where it was seen within the active site of the *C. violaceum* omega transaminases.

From what has been seen so far, differential scanning fluorometry appears to be a potential tool in protein crystallography. The results from it appear to be positive for it showed the current Tris buffer to stabilise the *S. avermitilis* omega transaminase protein the least of those tested and would explain the disordered crystals that have been obtained up until now. To confirm this, crystallisation trials will need to be performed with one of the buffers with the highest Tm values and see if there is any improvement in crystal disorder.

The F89A mutant *C. violaceum* omega transaminase had some success in using β alanine as a substrate but only at a slow rate. It is clear that increasing the flexibility of the carboxyl binding group R416 and creating more room in the active site where the F89 residue was located seems to allow a small amount of activity towards β alanine but it is insufficient to confer full activity towards this substrate. Further mutations will be necessary to allow optimal use of β -alanine. An amino acid target identified in this project would be the F88 residue, whose side chain is located close to the location where β -alanine would need to be located upon binding to the R416 to also bind the PLP co-factor.

5.2. Future Work

- Crystallising *S. avermitilis* omega transaminase within one of the buffers that provided the highest Tm values from the differential scanning fluorometry to see if there is any improvement the crystals formed.
- The F89A mutant from *C. violaceum* omega transaminase should be screened for changes in activity towards other substrates and see what effects it has. Also more mutants will need to be generated if β-alanine is to be used as a substrate by this enzyme.
- The *D. geothermalis* omega transaminase needs a new protocol to be made for the purification of the enzyme without the standard Nickel affinity chromatography. Hydrophobic interaction chromatography has already proven a viable step. All that is most likely required is an alternative to ion exchange chromatography as a purification step before gel filtration chromatography. A new construct with the His-tag on the C-terminal end of the protein could be considered also.
- The remaining crystals of *P. aeruginosa* omega transaminase from the cocrystallisation trials need to go to the Diamond Synchrotron for X-ray diffraction to try and find a crystal from the gabaculine co-crystallisation experiment that will diffract well.

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