Structural Determinants of the β-Selectivity of a Bacterial Aminotransferase

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Background: β-Transaminases are promising biocatalysts for the synthesis of β-amino acids.

Results: First 3D structures were obtained of a native β-transaminase, and complexes with a keto-acid and two covalently bound β-amino acids.

Conclusion: Dual functionality of the carboxylate and side-chain binding pockets allows binding of β- and α-amino acids.

Significance: These structures may facilitate the development of improved β-amino acid biocatalysts.

SUMMARY

Chiral β-amino acids occur as constituents of various natural and synthetic compounds with potentially useful bioactivities. The pyridoxal-5'-phosphate dependent (S)-selective transaminase from *Mesorhizobium* sp. LUK (*MesAT*) is a fold type I aminotransferase that can be used for the preparation of enantiopure β-phenylalanine and derivatives thereof. Using X-ray crystallography, we solved structures of *MesAT* in complex with (S)-β-phenylalanine, (R)-3-amino-5-methylhexanoic acid, 2-oxoglutarate, and the inhibitor 2-aminooxyacetic acid, which allowed us to unveil the molecular basis of the amino acid specificity and enantioselectivity of this enzyme. The binding pocket of the side chain of a β-amino acid is located on the 3'-O side of the PLP cofactor. The same binding pocket is utilized by *MesAT* to bind the α-carboxylate group of an α-amino acid. A β-amino acid thus binds in a reverse orientation in the active site of *MesAT* as compared to an α-amino acid. Such a binding mode has not been reported before for any PLP-dependent aminotransferase and shows that the active site of *MesAT* has specifically evolved to accommodate both β- and α-amino acids.

INTRODUCTION

β-Amino acids occur as precursors of many natural and synthetic compounds that display a wide range of pharmacological activities. Altering and improving the pharmacological properties of these compounds critically depends on the availability of β-amino acids and their derivatives as building blocks. Therefore, several strategies for the synthesis of β-amino acids have been explored over the years, involving either synthetic (1,2) or combined chemo-enzymatic (3) methods. However, fully enzyme-based synthesis methods have clear advantages over synthetic or chemo-enzymatic methods (4) and have the potential to increase the feasibility of biocatalytic or fermentative routes towards β-amino compounds.

Pyridoxal-5'-phosphate (PLP)1 dependent aminotransferases (also called transaminases) are attractive for the production of amino acids since they have a broad substrate range, can be highly enantioselective, show a high catalytic activity, and are relatively stable (5). Aminotransferases catalyze the transfer of an amino group from an amino compound to a keto-acid. In the first half reaction, the amino group of the amino compound substitutes the covalent Schiff base linkage, or imine bond, between the ε-amino group of a lysine

1 Abbreviations: PLP, pyridoxal-5'-phosphate; PMP, pyridoxamine; MesAT, aminotransferase from *Mesorhizobium* sp. strain LUK; (S)-β-Phe, (S)-β-phenylalanine; AroAT, aromatic amino acid aminotransferase from *Paracoccus denitrificans*; AOA, 2-aminooxyacetic acid; L-PPG, 2-amino-4-pentynoic acid.
and the C4A-atom of the PLP cofactor, generating an external aldime (Fig. 1). Lysine-assisted transfer of a proton from the external aldime to the C4A-atom of the cofactor results in a ketimine intermediate, which is hydrolyzed to yield pyridoxamine (PMP). Subsequently, in the second half reaction, the amino group of PMP is transferred to a keto-acid which generates a new amino compound (Fig. 1) (6).

The production of \( \beta \)-amino acids using PLP-dependent aminotransferases has been demonstrated for an \( \alpha \)-transaminase from *Polaromonas* sp. JS666 (7) and a \( \beta \)-transaminase from *Mesorhizobium* sp. LUK (MesAT)(8). The genes of these two enzymes were successfully cloned and expressed in *E. coli* BL21(DE3) using pET-based expression systems (7,8) and MesAT has been crystallized (9). MesAT is an enzyme of 445 amino acids with an \( M_w \) of 45 kDa, which forms dimers in solution and which accepts \( \beta \)-as well as \( \alpha \)-amino acids (8,10). The enzyme can convert the aliphatic \( \beta \)-amino acids (R)-3-amino-5-methylhexanoic acid and (R)-3-aminobutyric acid as well as the aromatic \( \beta \)-amino acid (S)-\( \beta \)-phenylalanine ((S)-\( \beta \)-Phe) to the corresponding \( \beta \)-keto acids using pyruvate or 2-oxoglutarate as the amino-acceptor. In the reverse reaction, MesAT can be used to produce (S)-\( \beta \)-Phe from its \( \beta \)-keto acid esthylester in a coupled enzyme reaction containing a lipase to generate the keto-acid in situ and rac-3-aminobutyric acid as amino-donor (8). Unfortunately, the more stable keto-ester is a very poor substrate for aminotransferase-mediated conversion to (S)-\( \beta \)-Phe.

Information on 3D-structures of \( \beta \)-aminotransferases is currently lacking. To enable rational protein engineering approaches for improving the activity of the enzyme for application in the biosynthesis of \( \beta \)-amino acids, we have elucidated the crystal structure of MesAT in the native state, as well as in complex with \( \beta \)-amino acids, a keto-acid, and an inhibitor. The structure of the enzyme conforms to a fold type I aminotransferase structure, but the hydrophobic binding pocket is located on the 3'-O side of the PLP cofactor rather than on its phosphate side as found in other aminotransferases. On the other hand, an \( \alpha \)-amino acid binds in a normal orientation, with the \( \alpha \)-carboxylate on the 3'-O side and the side chain of the amino acid on the phosphate side of the PLP cofactor. The architecture of the active site explains how MesAT can accept \( \beta \)-as well as \( \alpha \)-amino acids (8), while the aromatic \( \alpha \)-amino acid aminotransferase AroAT (11) and most other aminotransferases only accept \( \alpha \)-amino acids. The structure also explains the stereopreference of the enzyme for \( \alpha \)- and \( \beta \)-amino acids.

**EXPERIMENTAL PROCEDURES**

Protein expression and purification—The MesAT gene was codon optimized for *E. coli*, synthesized by DNA2.0 Inc., and cloned into the expression plasmid pET28b+ with an N-terminal His\(_{6}\)-tag using NdeI/HindIII restriction sites. After transformation into *E. coli* strain BL21(DE3) cells were grown at 37 \( ^\circ \)C in 1.4 l TBS medium (Terrific Broth Sorbitol (12)) containing 50 \( \mu \)g/ml of kanamycin. Protein expression was induced with 0.4 mM IPTG (isopropyl-\( \beta \)-D-1-thiogalactopyranoside) when the optical density of the culture at 600 nm reached 0.6-0.8, and the temperature was adjusted to 17 \( ^\circ \)C. The cells were grown for another 48 h, then harvested and resuspended in a buffer containing 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 1 mg of DNaseI (Roche) and a Complete EDTA-free protease inhibitor tablet (Roche). The cells were disrupted at 4 \( ^\circ \)C by sonication followed by centrifugation for 1 h at 31,000 \( \times \) g at 4 \( ^\circ \)C.

The supernatant was applied to a HisTrap HP affinity chromatography column (GE Healthcare) and, after washing, MesAT was eluted with 15 column volumes of a linear gradient of 20 – 500 mM imidazole in elution buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl). The fractions corresponding to the peak were pooled, concentrated (Ultracel 30K MWCO, Amicon) and applied to a Superdex 200 10/300 GL size exclusion chromatography column (GE Healthcare), equilibrated in 20 mM Tris-HCl, pH 8.0, containing 200 mM NaCl. After elution the fractions corresponding to the protein peak...
were pooled, concentrated (Amicon) and dialyzed overnight against a buffer containing 20 mM Tris-HCl, pH 7.5. The sample was subsequently concentrated to 10 mg/ml as judged from a protein assay and the purity of the sample was checked with silver-stained SDS-PAGE gels using a Phast system (GE Healthcare).

**Mutagenesis**—The R412A mutant gene of *MesAT* (Supplementary Table S2) was constructed by site-directed mutagenesis (QuikChange, Stratagene) and transformed into *E. coli* DH5α ElectroMAX electrocompetent cells (Invitrogen). For overexpression *E. coli* BL21(DE3) was used. The mutant construct was confirmed by sequence analysis (GATC Biotech).

**Protein crystallization**—A Mosquito crystallization robot (TTP LabTech) was used to search for suitable crystallization conditions. Crystallization experiments were set up at 20 °C. Crystals were found in a JCSG+ Suite (QIAGEN) condition containing 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, 8% v/v ethylene glycol and 10% w/v PEG 8K. After optimization, it was found that this was also the optimal condition for crystal growth, with crystals reaching sizes of 80x50x30 µm. Crystals were transferred to a cryoprotection solution consisting of mother liquor with 20% v/v ethylene glycol. This was done in 4 steps of 5 min each, starting with a solution containing 2% (v/v) ethylene glycol, followed by solutions of 5, 10 and finally 20% (v/v) ethylene glycol. Crystals from this last solution were cryo-cooled in liquid nitrogen. For amino acid binding studies, the same steps were followed, but with the cryoprotection solutions supplemented with 2, 5, 10 and 20 mM (S)-β-Phe (Peptech Corp.), (R)-3-amino-5-methylhexanoic acid (Fluorochem), 2-oxoglutarate disodium salt (Fluka), or 2-aminoxyacetic acid (AOA) (Aldrich).

**Diffraction data collection and processing**—Diffraction data were collected at beam lines ID14-1 and ID14-2 of the European Synchrotron Radiation Facility (ESRF, Grenoble) and at beam line X13 of the EMBL outstation at the Deutsches Elektronen-Synchrotron (DESY, Hamburg). Reflections were indexed and integrated using XDS (13), and scaling and merging of the data was done with the program SCALA (14) from the CCP4 software suite (15). Phaser (16) was used for molecular replacement with a mixed input model generated by the FFAS03 server (17) on the basis of the structures of glutamate-1-semialdehyde-2,1-aminomutase from *Thermus thermophilus* HB8 (PDB entry 2E7U; Mizutani, H., Kunishima, N., RIKEN Structural Genomics/Proteomics Initiative, to be published), D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST-201 (PDB entry 2CY8; Kongsaeree, P., Shirouzu, M., Yokoyama, S., RIKEN Structural Genomics/Proteomics Initiative, to be published) and 4-aminobutyrate aminotransferase from pig (PDB entry 1OHV (18)). The resulting model was subjected to successive rounds of automatic model building with ARP/wARP (19) followed by manual model building in Coot (20). Refmac5 was used for refinement of the atomic coordinates and atomic B-factors (21). Data collection and refinement statistics are given in Supplementary Table S3. After refinement the model was validated with MolProbity (22). Stereocnical restraints for the amino acid analogues were generated using the PRODRG2 server (23). RMSD values were calculated with the RMSDcalc tool of the CaspR server (24) and structural homologues of *MesAT* were obtained from the Dali server (25). PISA from the CCP4 software suite was used for protein interface analysis (26). Simulated annealing composite omit maps were generated with PHENIX (27). Chemical structure drawings were made using the ChemDraw program (CambridgeSoft) and PyMOL (28) was used for making images of the protein structure.

Atomic coordinates and structure factors have been deposited in the Protein Data Bank, [www.pdb.org](http://www.pdb.org), with PDB codes 2YKU, 2YKY, 4AO4, 2YKX and 2YKV (Supplementary Table S3).

**Enzyme assay and analytical methods**—Aminotransferase assays were performed with 10 mM amino donor ((S)-β-phenylalanine), 10 mM amino acceptor (pyruvate) and enzyme at 37 °C in 50 mM MOPS buffer, pH 7.6, containing 50 µM PLP (Acros Organics). Samples were taken at different times and treated according to the following procedure: to 50 µl of sample, 50 µl of a 2M HCl was added to quench the reaction. The
sample was left on ice for 5 min and neutralized by adding 45 µl of 2M NaOH followed by adding 50 µl of demineralized water. In an HPLC autosampler (Jasco), 1 µl sample was mixed with 2 µl of an ortho-phthalaldehyde (Sigma) (OPA)-solution [15 mg of OPA was dissolved in 50 µl absolute ethanol, which was then mixed with 4.42 ml 0.4 M sodium borate (pH 10.4), 15 µl 30% w/v Brij 35 (Fluka) and 11 µl β-mercaptoethanol] and 5 µl 0.4 M sodium borate (pH 10.4). The OPA-derivatized samples were analysed by HPLC using a C18 Alltech Adsorbosphere 5u column (5 µm, 4.6 mm x 100 mm) using a Jasco HPLC system. Separation of OPA-derivatized imines was achieved at room temperature at a flow rate of 1 ml/min using a gradient of eluent A (5% THF in 20 mM sodium acetate, pH 5.5) and eluent B (99% pure CH3CN) as follows: start with 100:0 A:B for 5 min; in 7 min from 100:0 to 80:20 A:B; continue with 80:20 for 4 min, 8 min from 80:20 to 40:60; continue with 40:60 for 6 min; then change from 40:60 to 100:0 in 2 min and finally continue for 5 min at 100:0 for re-equilibration of the column. The eluate was analysed by UV (338 nm) using a Jasco UV-2075 Plus detector and with a Jasco FP-920 fluorescence detector (350 nm excitation and 450 nm emission). Retention times for derivatized L-α-alanine and (S)-β-phenylalanine were 7.7 min and 23.2 min, respectively. One unit of enzyme activity is defined as the amount of enzyme that achieves at room temperature at a flow rate of 1 ml/min using a gradient of eluent A (5% THF in 20 mM sodium acetate, pH 5.5) and eluent B (99% pure CH3CN) as follows: start with 100:0 A:B for 5 min; in 7 min from 100:0 to 80:20 A:B; continue with 80:20 for 4 min, 8 min from 80:20 to 40:60; continue with 40:60 for 6 min; then change from 40:60 to 100:0 in 2 min and finally continue for 5 min at 100:0 for re-equilibration of the column. The eluate was analysed by UV (338 nm) using a Jasco UV-2075 Plus detector and with a Jasco FP-920 fluorescence detector (350 nm excitation and 450 nm emission). Retention times for derivatized L-α-alanine and (S)-β-phenylalanine were 7.7 min and 23.2 min, respectively. One unit of enzyme activity is defined as the amount of enzyme that produces 1 µmol/min of alanine from 10 mM pyruvate (sodium salt; Fluka) and 10 mM (S)-β-phenylalanine (Acros Organics).

To determine the $V_{max}$ and $K_m$ values of the R412A mutant of MesAT, initial rate assays were done with varying concentrations of (S)-β-Phe or pyruvate, fixing the non-variant substrate at 10 mM. The reactions were started by adding 680 µg of purified protein, and incubated at 37 °C.

Inhibition studies were performed by pre-incubating the reaction mixture, lacking (S)-β-Phe but containing 40 µg of wild-type enzyme, with 5 mM 2-aminoxyacetic acid (AOA) (Aldrich) or 5 mM DL-propargylglycine (2-amino-4-pentynoic acid, PPG) (Sigma-Aldrich) for 5 min, after which (S)-β-Phe (PepTech. Corp.) was added.

RESULTS

Overexpression, purification and enzyme activity measurements of wild-type and R412A MesAT—Previously, an overexpression system of MesAT was reported that gave a yield of 1.4 mg pure protein per liter of culture (8). To obtain enhanced expression, we used a codon-optimized synthetic gene that was equipped with an N-terminal His tag, cloned it under control of the T7 promoter in a pET vector, and cultivated the transformed E. coli BL21(DE3) cells in TBS medium at 17 °C. This resulted in an expression level that allowed the isolation of about 10 mg of pure enzyme per liter of culture (Supplementary Table S1). The specific activity of the purified wild-type enzyme was 1.6 U • mg⁻¹, which is similar to what was reported earlier (8).

Structure determination of MesAT—The holo-enzyme crystallizes in space group C2 with three molecules (chains A, B and C) per asymmetric unit. The 3D-structure of MesAT was elucidated at 2.5 Å resolution by molecular replacement and refined at 1.65 Å resolution. No density is defined for the first 30 N-terminal residues and the C-terminal residue M445. The three molecules are very similar to each other with RMSD values of Cα atom positions of ~0.2-0.25 Å. They form 1½ dimer in the asymmetric unit; one dimer consists of chains A and B (Supplementary Figure S1), the other dimer is made up of chain C and a chain C from a neighboring asymmetric unit related by crystallographic two-fold symmetry. Both dimers are very similar, with RMSD values in the order of 0.2 Å (Cα atoms). The two chains in the dimer interact tightly, burying a surface area of about 4400 Å², which is a quarter of their total surface area. The presence of dimers in the crystal is in agreement with the occurrence of MesAT dimers in solution (10).

The structure of the MesAT monomer—The MesAT monomer has a curved shape and consists of a PLP-binding domain (residues 112-334) and a domain formed by the N- and C-termini of the polypeptide chain (NC-domain; residues 1-111 and 335-445) (Supplementary Figure S1). These two domains line a cleft into which the PLP cofactor protrudes. The monomer contains 11 α-helices (of at least 2 or more turns) and 12 β-strands that form a mixed central 7-stranded β-sheet in the large domain and a 3-stranded as well as a 2-stranded anti-parallel β-sheet in the NC-domain (Supplementary Figure
S1). The overall structure is similar to that of aspartate aminotransferase, the archetypical representative of fold type I aminotransferases (Z-score 21, RMSD of 4.2 Å for 309 Ca atoms, 16 % sequence identity, PDB entry 1BGK) (29,30) and the aromatic α-amino acid aminotransferase from *Paracoccus denitrificans* (AroAT) (Z-score 21, RMSD of 4.8 Å for 326 Ca atoms, 15 % sequence identity, PDB entry 1AY4) (11,31), which is specific for L-α-phenylalanine.

**The PLP cofactor**—Each MesAT monomer contains a pyridoxal-5’-phosphate (PLP) cofactor, covalently anchored via an imine bond (Schiff base) to the ε-amino group of residue K280. The amide protons of residues G145, T146 and T314 (from monomer B in case of the AB dimer) anchor the phosphate group of the PLP cofactor to the protein backbone. The pyridine ring of the PLP cofactor is stacked between residues V255 (at the si-face of the pyridine ring (32)) and Y172 (at the re-face of the pyridine ring). The nitrogen atom of the pyridine ring is at hydrogen bonding distance to residue D253. These interactions keep the PLP cofactor secured in the active site.

**The binding of (S)-β-phenylalanine**—To analyze how (S)-β-Phe binds in the active site, a crystal structure of MesAT with bound (S)-β-Phe was solved at 1.7 Å resolution. (S)-β-Phe binds covalently to the PLP cofactor via its β-amino group, substituting the imine bond between the ε-amino group of residue K280 and the C4A atom of the PLP cofactor (Fig. 2A). It binds with an estimated occupancy of about 80% in the three subunits. The carboxylate group of (S)-β-Phe has a salt bridge interaction with the Nε- and Nη2-atoms of R54. The aromatic ring of (S)-β-Phe is bound between the side chains of Y89 and Y172 with edge-to-face interactions (Fig. 2A) and has also van der Waals interactions with residues I56, A225, M256 and M414 from monomer A as well as with residue A312 from monomer B; these residues line a hydrophobic binding pocket that is capped by residue R412 (monomer A). Thus, residues from both monomer A and monomer B contribute to the binding of the aromatic side chain of (S)-β-Phe. The side chain amino group of K280 is close to the Cβ (R)-proton of (S)-β-Phe (3.3 Å), in agreement with its role in proton transfer (Fig. 1). The binding of (S)-β-Phe does not induce large-scale conformational changes or domain movements in MesAT. Only local conformational changes have occurred (see Discussion).

In monomer A, a second (S)-β-Phe molecule is present in a surface pocket, with its aromatic ring stacked between residues L269 and L368. Its carboxylate and amino groups point into the solvent and do not interact with the protein. However, in the surface pockets of monomers B and C electron density is only present for the aromatic ring of an (S)-β-Phe molecule, but not for the amino- and the carboxylate groups. From this we conclude that (S)-β-Phe binds non-specifically in this surface pocket.

**The binding of (R)-3-amino-5-methylhexanoic acid**—To investigate how an aliphatic β-amino acid such as (R)-3-amino-5-methylhexanoic acid (8) binds in the active site, a 1.95 Å resolution crystal structure was determined of MesAT in complex with this compound. (R)-3-amino-5-methylhexanoic acid binds covalently to the PLP cofactor via its β-amino group, substituting the imine bond between the ε-amino group of residue K280 and the C4A atom of the PLP cofactor (Fig. 2B). It binds with an estimated occupancy of about 90% in the three subunits. The carboxylate group of (R)-3-amino-5-methylhexanoic acid has a salt bridge interaction with the Nε- and Nη2-atoms of residue R54, and the aliphatic side chain (atoms C4, C5 and C6) binds in the hydrophobic binding pocket where also the aromatic side chain of (S)-β-Phe was observed to bind (see above). Binding of (R)-3-amino-5-methylhexanoic acid only produces limited local conformational changes in MesAT.

**The binding of 2-oxoglutarate**—To analyze how a keto-acid binds in the active site of MesAT we determined the crystal structure of the enzyme with bound 2-oxoglutarate at 1.85 Å resolution (Fig. 2C). The compound binds non-covalently in the active site. Its α-carboxylate has a bidentate salt-bridge interaction with the Nη1- and Nη2-atoms of R412. The γ-carboxylate binds with one of its oxygen atoms to the Nε- and Nη2-atoms of R54; the other oxygen atom is not involved in hydrogen bond formation. The keto-oxygen atom of 2-oxoglutarate is located at 3.0 Å from the nitrogen atom of the K280 side chain, which is covalently bound to the C4A atom of the PLP cofactor. The binding of 2-oxoglutarate thus
leaves the internal aldimine intact. The orientation of 2-oxoglutarate in the active site of MesAT suggests that amino group transfer from PMP results in the synthesis of L-glutamate, in agreement with chiral HPLC analysis of the product of a reaction with (S)-β-Phe as amino-donor and 2-oxoglutarate as amino-acceptor (data not shown). Binding of 2-oxoglutarate does not result in large-scale conformational changes of MesAT. However, residue R412 reorients its side chain such that its Nη1- and Nη2-atoms can make a salt bridge interaction with the α-carboxylate of 2-oxoglutarate. This reorientation of the arginine side chain in response to the binding of an amino acid in the active site of an aminotransferase is referred to as the ‘arginine switch’ (33).

The importance of R412 for activity was confirmed by mutagenesis. The protein yield of the R412A variant was similar to that of the wild-type enzyme. The \( k_{\text{cat}} \) values of the R412A mutant were significantly lower than those of the wild-type enzyme (Table 1). Furthermore, the \( K_m \) value for pyruvate had increased by a factor of 28, whereas for (S)-β-Phe it had decreased by a factor of 4. The relationship between the reaction rate of the R412A mutant and the substrate concentration indicated substrate inhibition, as observed in the wild-type enzyme (34) (Table 1).

The binding of 2-aminoxyacetic acid (AOA)—AOA (35) and 2-amino-4-pentyoic acid (L-PPG) (36,37) are known inhibitors of aminotransferase activity. Pre-incubation of MesAT with 5 mM of these inhibitors resulted in an activity decrease of 10 % upon treatment with L-PPG and a 96 % decrease upon treatment with AOA. Analysis of the 1.9 Å resolution crystal structure of MesAT with bound AOA (Fig. 2D) shows that the amino group of AOA binds covalently to the C4A atom of PLP, as has also been observed for the interaction of AOA with aspartate aminotransferase (38). The ether oxygen atom (OX1) is close to K280 (2.9 Å) and the carboxylate group binds via a salt bridge to the Nε- and Nη2-atoms of R54. The binding of AOA to the PLP cofactor is irreversible; the amine-OX1 bond cannot be weakened by residue K280. Binding of AOA to the PLP cofactor thus prevents PMP formation and thereby inhibits aminotransferase activity. No significant conformational changes are observed upon AOA binding.

**DISCUSSION**

*The structure of MesAT*—The structure of MesAT presented here is the first structure of a transaminase with specificity towards β-amino acids as well as α-amino acids. The structure of the enzyme is similar to that of aspartate aminotransferase (39), the archetypical fold type I aminotransferase. The enzyme assembles into a homo-dimer in which residues from both monomer A and B contribute to the binding of amino acid and their respective oxo-acid substrates. In contrast to aspartate aminotransferase, the binding of ligands does not induce large domain movements. Structural changes that do occur in MesAT upon substrate/inhibitor binding are localized to the active site and consist of a 16° rotation of the pyridine ring of the PLP cofactor upon formation of the external aldimine. This rotation liberates K280 and allows it to function as the proton transferring lysine. Another structural change is the rearrangement of the R412 side chain, also known as the arginine switch (33), upon binding of 2-oxoglutarate (mentioned above).

Different binding modes of (S)-β-phenylalanine, (R)-3-amino-5-methylhexanoic acid and 2-oxoglutarate—The way in which 2-oxoglutarate binds in the active site of MesAT and the manner in which (S)-β-phenylalanine and (R)-3-amino-5-methylhexanoic acid bind are very different. The side chains of (S)-β-Phe (Fig. 2A) and (R)-3-amino-5-methylhexanoic acid (Fig. 2B) bind in a pocket on the 3'-O side of the PLP cofactor, which we denote the O-pocket. However, the side chain of the α-keto acid 2-oxoglutarate, a model for the α-amino acid L-glutamate, binds with its γ-carboxylate group on the phosphate side of the PLP cofactor (Fig. 2C), in the P-pocket. Thus, MesAT has two distinct side chain binding pockets, one for the side chains of β-amino acids (O-pocket, Fig. 3) and one, on the other end of the active site, for the side chain of α-keto acids and presumably also α-amino acids (P-pocket, Fig. 3). The enzyme has also two carboxylate binding pockets; one, involving R54 in the P-pocket, binds the α-carboxylate group of β-amino acids and of AOA, a β-alanine mimic (Fig. 2D), while the other,
which contains R412 in the O-pocket, binds the α-carboxylate of α-keto acids such as 2-oxoglutarate (Fig. 2C). As a consequence, an α-amino acid and a β-amino acid have very different binding modes in MesAT (Fig. 3A,B).

Intriguingly, the O-pocket that binds the aliphatic and hydrophobic side chain of β-amino acids also binds the α-carboxylate group of α-keto acids and presumably also of α-amino acids. This dual functionality is made possible by a switch in position of the R412 side chain, the arginine switch residue (33). When a β-amino acid binds, the R412 side chain is oriented away from the active site, providing space in the O-pocket for the hydrophobic side chain of the β-amino acid (Figs. 2A,B). This orientation of R412 is stabilized by a hydrogen bond of its side chain Nη-atom to the carbonyl oxygen atom of A225 (monomer B, not shown). In contrast, upon 2-oxoglutarate binding, the side chain of R412 switches back towards the hydrophobic O-pocket (Fig. 2C); the hydrogen bond with A225 is broken and R412 now has a salt-bridge interaction with the α-carboxylate of 2-oxoglutarate. In this way the R412 side chain allows the enzyme to accept both α-keto/α-amino acids and aliphatic- or aromatic β-amino acids in the same active site.

Because of the dual functionality of the active site pockets of MesAT we prefer to use O- and P-pocket, rather than L- and S-pocket nomenclature, which reflects the presumed size of these pockets (40).

For aminotransferase activity R412 is virtually essential, as reflected by the 870-fold reduction of the catalytic efficiencies for pyruvate and (S)-β-Phe, respectively, upon mutation of this residue to Ala (Table 1). Whereas the decreased apparent \( K_m \) for (S)-β-Phe can be due to the reduced catalytic rate at 10 mM pyruvate, the increased \( K_m \) for pyruvate strongly supports that R412 is important for binding pyruvate through electrostatic interactions with its carboxylate.

Comparison of MesAT and AroAT—To investigate how MesAT differs from an aminotransferase that only accepts α-amino acids, the MesAT structures were compared with that of the aromatic amino acid aminotransferase AroAT from P. denitrificans (11). In both enzymes the PLP cofactor has a similar position and orientation. In contrast to MesAT, AroAT has a single α-carboxylate binding pocket and charged as well as uncharged α-amino acids bind with their α-carboxylate groups to R386 in the O-pocket (Fig. 3C,D). In MesAT, the α-carboxylate group of (S)-β-Phe binds to R54 in the P-pocket, while the side chain of (S)-β-Phe is bound in the O-pocket (Fig. 3A). As a consequence, in MesAT, β-amino acids bind in a reverse orientation in comparison to the substrates of AroAT (Fig. 3A,C,D). Moreover, the arginine switch of MesAT (R412) is located in the O-pocket of the enzyme, where it binds the α-carboxylate group of an α-amino acid, while in AroAT the arginine switch (R292), is located in the P-pocket of the enzyme and binds the side chain carboxylate group of α-amino/α-keto acids (Fig. 3A-D). These differences between MesAT and AroAT show that, while these enzymes share the same fold, the architecture of their active site is very different. In MesAT the active site architecture has evolved to accommodate β-amino acids, while retaining the ability to accommodate α-amino/α-keto acids.

The covalent adducts in the active site of MesAT represent external aldimine intermediates—Structures obtained from crystals soaked with (S)-β-Phe and (R)-3-amino-5-methylhexanoic acid show that covalent PLP-β-amino acid adducts have formed in the active site of MesAT (Fig. 2A,B). These adducts represent external aldimine intermediates since the Nβ-, Cα-, Cβ- and Cγ-atoms are not coplanar, as would occur in the ketimine intermediate (Fig. 1). The non-coplanarity of the Nβ-, Cα-, Cβ- and Cγ-atoms suggests that the reaction has stopped before abstraction of the Cβ-proton.

Different explanations for the trapping of the external aldimine trapping may be considered. Proton abstraction is most efficient if, in the transition state, the bond to be broken is oriented perpendicular to the plane of the PLP ring system (41). The crystal structures indicate that the Cβ-proton is indeed nearly perpendicular to the PLP plane, deviating 20-30° from the perpendicular position. Such a deviation is probably not sufficient to fully prevent proton abstraction.

Another explanation may be related to the observation that in none of the external aldimine intermediate bound structures density is present for a hydrolytic water molecule near the Cβ-atom that could convert the ketimine intermediate into...
the pyridoxamine intermediate (Fig. 1). The equilibrium of the aminotransferase reaction in the crystal structure of MesAT may thus lie towards the external aldimine intermediate rather than the ketimine intermediate which could explain why the external aldimine intermediates of (S)-β-Phe and (R)-3-amino-5-methylhexanoic acid are trapped.

Enantioselectivity of MesAT—MesAT is enantioselective towards the β-amino acids (S)-β-Phe, (R)-3-amino-5-methylhexanoic acid and (R)-3-aminobutyric (8). These preferred enantiomers have the same stereoconfiguration of functional groups on the Cβ-atom as (S)-β-Phe. The preference for these enantiomers can be fully explained by the architecture of the active site which forces these substrates to bind in an orientation in which the carboxylate group binds to R54 in the P-pocket and the side chain in the O-pocket followed by addition of an amino group at the si-face of the β-carbon of the β-keto acid.

The insights obtained from the 3D-structure of MesAT on the enzyme’s interaction with α- and β-amino/keto acids may facilitate structure-based protein engineering efforts to enhance the biocatalytic potential of β-transaminases for the production of β-amino acids of pharmacological interest.

REFERENCES


28. The PyMOL molecular graphics system, version 1.2r3pre. Schrodinger, LLC

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FOOTNOTES

These two authors contributed equally to this study.

FIGURE LEGENDS

Figure 1. Half reactions catalyzed by MesAT, a β-specific PLP-dependent transaminase. An amino acid ((S)-β-Phe) enters the active site of the enzyme (1) and an external aldimine is generated. K280, a base, abstracts the Cβ-proton (2) and transfers the proton to the C4A-atom (1,3-prototropic shift, not shown), which results in the ketimine intermediate. Hydrolysis of the ketimine intermediate results in the formation of pyridoxamine phosphate (PMP) and a keto-acid (3-oxo-3-phenylpropanoic acid) (3).

Figure 2. Stereo figures of simulated annealing composite 2mFo-DFc omit maps contoured at 1σ. A) (S)-β-Phe (magenta) bound to the PLP cofactor. For clarity residue Y172 has been omitted from this figure as well as from figures 2B, 2C and 2D. B) (R)-3-amino-5-methylhexanoic acid (magenta). For clarity residue A312 has been omitted from this figure as well as from figures 2C and 2D. C) 2-oxoglutarate (magenta) bound in the active site of MesAT [LLP = (2-lysine(3-hydroxy-2-methyl-5-phosphonoxyethyl-pyridin-4-ylmethane)], the internal aldimine. D) 2-aminooxycetic acid (magenta), a β-alanine mimic, bound in the active site of MesAT.

Figure 3. Comparison of the active site architectures of the β-aminotransferase MesAT (left column) and the α-aminotransferase AroAT (right column). A) Surface rendition of the active site of MesAT bound with (S)-β-Phe (magenta) and B) with 2-oxoglutarate (magenta). C) Surface rendition of the active site of AroAT bound with 3-phenylpropionate (magenta) based on PDB entry 1AY8 (31) and D) with maleate (magenta) based upon PDB entry 1AY5 (31). The positions of the PLP cofactor as well as R412 and R386 in the active sites of MesAT and AroAT, respectively, are similar. R412 represents the arginine switch of MesAT, R292 represents the arginine switch of AroAT. LLP, the internal aldimine. P (P-pocket) and O (O-pocket), see text for explanation.
Figure 1.
Figure 2C, D.
Figure 3A,B,C,D.
Table 1. Specific activity at 37 °C of wild-type \textit{Mes}AT and the R412A mutant using (S)-β-Phe as amino-donor and pyruvate as amino-acceptor.

<table>
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<th>MesAT</th>
<th>Sp. act.</th>
<th>Rel. act.</th>
<th>$K_m$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
<th>$K_i$</th>
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<tr>
<td></td>
<td>(U • mg$^{-1}$)$^a$</td>
<td>(%)</td>
<td>(mM)</td>
<td>(s$^{-1}$)</td>
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<td>100</td>
<td>1.2$^b$</td>
<td>1.3</td>
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<td>3.2$^b$</td>
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<tr>
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<td>0.4</td>
<td>0.29</td>
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<td>2•10$^{-2}$</td>
<td>47</td>
<td>This study</td>
</tr>
</tbody>
</table>

$^a$ Abbreviations: Sp. act., specific activity; Rel. act., relative activity; WT, wild-type.

$^b$ Adopted from Kim and coworkers (8).