Biochemical properties of a *Pseudomonas* aminotransferase involved in caprolactam metabolism

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Abstract

The biodegradation of the nylon-6 precursor caprolactam by a strain of Pseudomonas jessenii proceeds via ATP-dependent hydrolytic ring-opening to 6-aminohexanoate. This non-natural ω-amino acid is converted to 6-oxohexanoic acid by an aminotransferase (PjAT) belonging to the fold type I PLP enzymes. To understand the structural basis of 6-aminohexanoate conversion, we solved different crystal structures and determined the substrate scope with a range of aliphatic and aromatic amines. Comparison with the homologous aminotransferases from Chromobacterium violaceum (CvAT) and Vibrio fluvialis (VfAT) showed that the PjAT enzyme has the lowest $K_M$ values (highest affinity) and highest specificity constant ($k_{cat}/K_M$) with the caprolactam degradation intermediates 6-aminohexanoate and 6-oxohexanoic acid, in accordance with its proposed in vivo function. Five distinct three-dimensional structures of PjAT were solved by protein crystallography. The structure of the aldime intermediate formed from 6-aminohexanoate and the PLP cofactor revealed the presence of a narrow hydrophobic substrate-binding tunnel leading to the cofactor and covered by a flexible arginine, which explains the high activity and selectivity of the PjAT with 6-aminohexanoate. The results suggest that the degradation pathway for caprolactam has recruited an aminotransferase that is well adapted to 6-aminohexanoate degradation.

Database

The atomic coordinates and structure factors Pseudomonas jessenii 6-aminohexanoate aminotransferase have been deposited in the PDB as entries 6G4B (E∙succinate complex), 6G4C (E∙phosphate complex), 6G4D (E∙PLP complex), 6G4E (E∙PLP∙6-aminohexanoate complex), and 6G4F (E∙PMP complex).

Abbreviations

AT, aminotransferase; AlaDH, alanine dehydrogenase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; 6-AHA, 6-aminohexanoic acid.

Introduction

Caprolactam is a bulk chemical mostly used for the industrial production of the polyamide nylon 6 [1]. This synthetic polymer has found widespread application in various industrial and household products, such as packaging materials, fibers and fabrics, utensils, and mechanical parts. In the nylon 6 manufacturing process, caprolactam undergoes a ring opening polymerization reaction at 240-270°C in the presence of water. After the polymerization process, several undesired side products remain, including the unreacted lactam and 6-aminohexanoate monomers, oligomers, and cyclic dimers [2]. Caprolactam and its side products should be removed before waste or wastewater is discharged into the environment, since release into natural water streams will

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threaten environmental quality and public health. To understand the environmental fate of nylon-related compounds and their biodegradation, several studies have been carried out aimed at isolating microorganisms that metabolize caprolactam and 6-aminohexanoate (cyclic) oligomers [3-6]. Some of these strains contain plasmids that harbour genes involved in caprolactam utilization, including genes encoding 6-aminohexanoate dimer hydrolase and 6-aminohexanoate cyclic dimer hydrolase, which are responsible for hydrolysis of amide bonds [5-7]. Whereas a complete degradation pathway is also suggested in the MetaCyc database (see URL http://MetaCyc.org) [8], most enzymes of the caprolactam catabolic route remained unidentified until two rather different aminotransferases that may catalyze 6-aminohexanoate deamination were recently discovered [9,10]. Our lab described the caprolactam-utilizing bacterium Pseudomonas jessenii strain OJG3, which produces an ATP-dependent lactamase that converts caprolactam to 6-aminohexanoate. This intermediate is converted by an aminotransferase that transfers the amino group to pyruvate and produces 6-oxohexanoic acid, which can be metabolized by β-oxidation via formation of adipic acid [10] (Fig. 1).

Aminotransferases (ATs) are pyridoxal 5'-phosphate (PLP)-dependent enzymes that catalyze the transfer of an amino group from a donor (e.g. an amino acid or amine) to an acceptor, which in vivo is most often pyruvate or 2-oxoglutarate [13,14]. After binding of the substrate, in the first half reaction its amino group is transferred to the PLP cofactor via formation of an imine (external aldimine) and proton shift, leading to a pyridoxamine (E-PMP) intermediate. Following release of the deaminated product as a ketone or aldehyde, in the second half reaction the enzyme binds the ketoacid acceptor and the amino group of PMP is transferred to this acceptor, again via imine formation and proton shift. The aminated product is released by cleavage of the Schiff base linkage under regeneration of enzyme-bound PLP. Based on crystal structures, PLP-dependent enzymes have been divided into different fold types, subgroups and classes, which to a certain extend correlates to reaction type or substrate scope [15-17].

Aminotransferases often belong to fold type I or fold type IV PLP enzymes [14-18]. Examples of well-studied fold type I aminotransferases are L-aspartate ATs [19], branched L-amino acid ATs, β-amino acid ATs [20,21] and the ω-aminotransferases (ωATs) from Vibrio fluvialis, Chromobacterium violaceum, Paracoccus denitrificans, and Ochrobactrum anthropi [22-26]. Fold type IV enzymes include D-amino acid ATs and (R)-amine selective ωATs [13-16,27,28]. Aminotransferases have also been classified in subgroups or classes, mainly based on substrate structural features, leading to a classification that partially agrees with grouping in fold types [15,17]. The subgroup AT-I aminotransferase are active with α-amino acids and thus include the PLP fold type I aspartate ATs and branched L-amino acid ATs. The original AT-II subgroup [15], later also called class III ATs, includes ω-aminotransferases, i.e. enzymes acting on non-α-amino acids [17]. The sequence suggests that the P. jessenii strain GO3 aminotransferase (PJAT) of the caprolactam catabolic pathway is most similar to these class III ATs [10], of which structures and sequences have been compared in detail [17,25].

Aminotransferases offer a diversity of realized and potential biotechnological applications. They usually have a high catalytic activity and do not require an external redox cofactor recycling system if an organic amine is used as the donor [22,29-32]. Process conditions for amination reactions catalyzed by enzymes are milder than in case of chemocatalytic incorporation of amine groups, which can make the use of aminotransferases attractive from an environmental point of

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Of particular interest is the production of chiral amines by asymmetric transformation of non-chiral keto-precursors. In such reactions, aminotransferases often show high regio-, enantio-, and chemoselectivity. Other attractive reactions are the terminal amination of aldehydes to produce amines, either in cascade conversions with free enzymes starting with ammonia and alcohol or by employing reactions with whole cells [34,35]. Recent work indicates that aminotransferases can be important for constructing artificial biosynthetic pathways, even leading to the biosynthesis of non-natural amines such as 6-aminohexanoate (6-AHA), the precursor of caprolactam [36]. Amino-hexanoic acid is also used as an antifibrinolytic drug in surgery. It can prevent excessive blood loss by inhibiting tissue plasminogen activation [37].

To discover an aminotransferase involved in caprolactam metabolism and examine its potential use in various enzymatic transformations of biotechnological interest, we investigated the caprolactam degradation pathway of *P. jessenii* GO3, using a proteomic and genomic approach [10]. The aminotransferase (PjAT) present in this strain acts in a catabolic pathway that involves compounds that are only known from chemical synthesis. The enzyme is examined here addressing the question if the enzyme is evolved to deaminate 6-aminohexanoic acid, a synthetic amino acid. The substrate range is determined and compared to that of related enzymes. We solved several crystal structures to explain the activity towards the biologically unknown substrate 6-AHA. We also examined the activity in the amination of 6-oxohexanoic acid and interpret the selectivity using 3D structures.

**Results**

**Catalytic properties of *P. jessenii* 6-aminohexanoate aminotransferase**

To overproduce the recently discovered *P. jessenii* aminotransferase involved in bacterial caprolactam degradation [10], we used a pET-based expression vector and *E. coli* strain Top10. After overnight growth under inducing conditions, a high-level expression of soluble enzyme was reached. Preparation of cell lysate by sonication, centrifugation and enzyme isolation by His-tag Ni-affinity chromatography yielded 34 mg of purified protein per L of culture. This material was used for activity profiling and crystallography. The dimeric enzyme (49.64 kDa subunits) showed activity towards 6-aminohexanoate (6-AHA) and 6-oxohexanoic acid, the aminated and deaminated intermediates of the caprolactam and 6-aminohexanoate oligomer degradation pathways (Fig. 1). With 2 mM 6-aminohexanoate and pyruvate as amino donor, an activity of 0.9 U/mg was found for the purified enzyme, corresponding to a *k*<sub>cat</sub> of 0.3 s<sup>-1</sup> (per subunit).

Compounds such as amino acids, aliphatic amines and aromatic amine compounds are of interest for the production of various pharmaceuticals, paintings, and crop protectants [38,39]. Therefore, specific activities of PjAT were measured using over 40 different amine substrates, including aromatic compounds, linear aliphatic compounds, and amino acids (Table 1). This activity screening was performed with spectrophotometric coupled-enzyme assays in which aminotransferase-mediated formation of L-alanine from pyruvate and 6-aminohexanoate was coupled to alanine dehydrogenase-mediated production of NADH. Data were compared to values...
measured with the aminotransferases from V. fluvialis (VfAT, 42% sequence identity) and C. violaceum (CvAT, 40% sequence identity).

The results (Table 1) showed that the PjAT enzyme, as well as CvAT and VfAT, had high activities with aromatic compounds carrying the amine functionality on short aliphatic side groups, e.g. α-methylbenzylamine ((S)-MBA and benzylamine. For the latter two enzymes this is in agreement with Kaulmann et al. [24] and Shin and Kim and coworkers [40,41] who also observed that activities of CvAT and VfAT with benzylamines were higher than with aliphatic amines. Nevertheless, several aliphatic amines and the ω-amino acids 4-aminobutanoic acid, 5-aminopentanoic acid and the obvious “natural” substrate 6-AHA also gave good activities, especially with PjAT and CvAT. Glycine and most other α-amino acids tested were not converted. We found that also CvAT had high activity with 6-AHA, higher than found earlier [24]. Primary alkylamines similar to 6-AHA but with the carboxylate group replaced by a methyl or alkylamines carrying a phenyl group (4-aminophenylbutane) were also well converted by all three enzymes (Table 1).

Some of the tested compounds were a poor substrate for all three ATs examined. Three β-amino acids were tested, but none was converted by any of the enzymes (Table 1). This clearly distinguishes these aminotransferases from the homologous PLP fold-type I β-amino acid aminotransferases discovered in bacterial cultures enriched with β-Phe as growth substrate [20,21,42]. Likewise, no good conversion of proteinogenic amino acids was found, in case of CvAT and VfAT agreement with Kaulman et al. [24] and Shin et al. [43].

To further examine the apparent activity differences, kinetic studies were performed with the aminated substrates that gave the best activities as well as with 6-oxohexanoate, the direct deamination product of 6-AHA in the caprolactam degradation pathway (Table 2). The results revealed that of the three enzymes PjAT has the highest affinities for 6-AHA and 6-OHA, with apparent $K_\text{M}$ values that were at least 7-fold lower for the amine substrate and 33-fold lower in case of the aldehyde when compared to values for CvAT and VfAT. The $k_\text{cat}$ values, on the other hand, were highest for CvAT, but the physiologically most relevant $k_\text{cat}/K_\text{M}$ value was best for PjAT, in accordance with its function in caprolactam degradation. Interestingly, PjAT had a lower apparent $K_\text{M}$ with 6-OHA than with the amine donor 6-AHA, indicating that the enzyme might be used for aldehyde amination, a reaction of importance for caprolactam production by metabolically engineered E. coli [36]. At high substrate concentrations, however, substrate inhibition was observed with all three enzymes when using the aldehyde as amine acceptor.

The results with other substrates confirmed that aryl-substituted alkylamines were well accepted by the three ω-aminotransferases (Table 2). With all amino compounds the highest $k_\text{cat}$ values were observed with CvAT, which was also found by Kaulmann et al. [24] when comparing CvAT and VfAT. Regarding affinities, it appeared that PjAT, which displayed the highest affinity for 6-AHA, showed the poorest affinity in case of the aromatic substrates. This gave a preference for 6-AHA, expressed as ratio of $k_\text{cat}/K_\text{M}$ values, which was 2- and 25-fold better for PjAT compared to CvAT and VfAT, respectively.

The high substrate affinity of PjAT was not only found with 6-AHA; also the shorter ω-amino acids 5-aminopentanoic acid and 4-aminobutanoic acid gave $K_\text{M}$ values that were at least 6-fold better in case of PjAT. The carboxylate group of the ω-amino acids seems to favor PjAT, since the higher affinity of this enzyme for ω-amino acids was not observed with other amines, such as 1-aminopentane and 2-phenylethylamine.

At high substrate concentration, substrate inhibition was observed with most of the aromatic compounds tested, with the exception of 2-phenylethylamine. This type of inhibition is in
line with previous reports demonstrating that aminotransferases are inhibited by high concentrations of an aromatic substrate [40]. It is likely due to binding of the amine substrate to the pyridoxamine intermediate rather than binding of the oxo-substrate that accepts the amine group.

Summarizing, the kinetic properties indicate that \( P_{jAT} \) is a more suitable catalyst for 6-AHA deamination than \( C_{vAT} \) or \( V_{fAT} \) in case of low substrate concentrations. This raises the question if the substrate’s carboxylate group located 5 carbons away from the reacting amine is involved in binding to the enzyme and in any evolutionary adaptation of \( P_{jAT} \) to 6-amino-hexanoate conversion.

Crystal structures

The structure of \( P_{jAT} \) was determined by protein crystallography using molecular replacement and refinement against 1.80 Å resolution diffraction data to an R-factor of 0.141 (Rfree = 0.166) with good stereochemistry (Table S1, Fig. 2). The protein is composed of two subunits forming a tight homodimer with an interface score calculated by PISA of 0.92 (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver). The dimeric \( P_{jAT} \) molecule has dimensions of 100 x 60 x 55 Å. The \( P_{jAT} \) structure is very similar to the structures of other PLP fold type I enzymes belonging to the \( \omega \)-aminotransferases, with DALI Z-scores over 20, rmsd values ~ 0.8-1.2 Å and sequence identities of 40-63%. These include the recently described \( \omega \)-aminotransferase from \( Ochrobactrum\ anthropi \) (5GHF); the putative aminotransferases from \( Mesorhizobium\ loti \) (3GJU, JCSG) and \( Silicibacter\ sp.\ TM1040 \) (3FCR, JCSG); the aminotransferase from \( Silicibacter\ pomeroyi \) (3HMU); the \( \omega \)-aminotransferase from \( Paracoccus\ denitrificans \) (4GRX), and the aminotransferases included in the activity comparison above \( C_{vAT} \) [4A6R] and \( V_{fAT} \) [3NUI] (Fig. 3). Only 32% identity is observed with a \( Pseudomonas\ aeruginosa \) \( \omega \)-aminotransferase (4B9B [45]) and a \( Pseudomonas\ putida \) \( \omega \)-aminotransferase (3A8U, unpublished).

All these PLP fold type I aminotransferases group into Clade 6c of the class II/III ATs, most of which are dimeric \( \omega \)-ATs with subunits consisting of two domains [25]. The smaller domain of \( P_{jAT} \) comprises residues 1 to 66 and 346 to 456. The large domain comprises residues 67 to 345 and contains most of the conserved residues, including residues that form the cofactor binding site.

The PLP-binding pockets of \( P_{jAT} \) are located at the dimeric interfaces and contain residues from both subunits. In the succinate and ammonium phosphate-grown crystals, a succinate or a phosphate ion, respectively, occupy the position of the phosphate moiety of the PLP in the homologous structures. The phosphates are hydrogen bonded to the backbone amides of Gly118 and Ser119, the backbone amide and sidechain of Thr324’, and five water molecules (Fig. 4A). The succinate molecule has the same interactions, including hydrogen bonds to the side chains of Ser286 and Lys287, to a water molecule and to a glycerol molecule (Fig. 4B). This glycerol molecule has hydrogen bonds to the carbonyl atoms of Asn116, Pro295 and to the carbonyl and side chain oxygens of Ser286. The glycerol occupies a small pocket shaped by Asn116, Ser292, Pro295 and Gly325’. In \( C_{vAT} \) and \( Silicibacter\ AT \) this pocket is occupied by a side chain of a Tyr, while it also exists in \( V_{fAT} \).
The PLP-binding pockets are well conserved between PLP fold type I class II or class III ATs [47]. In the PjAT-PLP structure there is continuous electron density from the side chain of Lys287 to the cofactor, revealing the internal aldimine adduct of PLP (Fig. 5A). The pyridine ring is sandwiched between the perpendicular ring of Tyr151 and the isopropyl group of Val260. The nitrogen of the ring has interaction with the side chain of the conserved Asp258. Additional density was observed close to the PLP. This was modelled as a glycerol and interacts with the ε-amino group of the catalytic Lys287, and side chains of Trp58, Tyr151, Ala230 and Arg417. Its position is different from the glycerol in the succinate experiment, being at the other side of Thr324’ at ~8 Å distance. The latter pocket is filled with water molecules in the PjAT-PLP structure.

Electron density maps of the PjAT-PLP crystal soaked with 6-AHA showed discontinuous residual density in subunit A between the ε-amino group of Lys287 and PLP, showing that the Schiff base bond is not present. Instead, additional density extending from the PLP carbonyl carbon was modelled as the 6-AHA–external aldimine adduct (Fig. 5B). In subunit B the unreacted internal aldimine was observed. The structure of the 6-AHA derived external aldimine revealed that the tunnel-like substrate-binding site is shaped by several mostly hydrophobic residues from both subunits. Tyr20, Met54, Leu57, Trp58, Tyr151, Leu164, Ile261, Ala230, Arg417, Met419, Phe86’, Ser87’ and Ala318’ provide a rather nonpolar substrate binding site. Upon binding of 6-AHA, Met419 obtains a double conformation with a new conformation pointing toward the substrate (Fig. 5B). The new position is possible by a switch of the side chain of Arg417, which is very mobile and adopts a different conformation in all five determined PjAT structures. High flexibility of an arginine corresponding to PjAT’s Arg417 is observed in other ATs as well [25,45,48]. In the 6-AHA aldimine adduct of PjAT, Arg417 forms a bidentate salt bridge with the carboxylate of 6-AHA through the Nɛ and N3 of the Arg417 side chain (3.8 and 3.7 Å distance), and there is an additional hydrogen bond of one of the carboxylate oxygens with the indole nitrogen of Trp58 (3.7 Å). Other contacts between substrate and enzyme are mainly hydrophobic, through the sidechains of Tyr20, Leu57, Phe86’ and Tyr151 (Fig. 5B). In electron density maps of the crystal soaking experiment with (S)-MBA, PMP was observed, but no density for (S)-MBA emerged in subunit A, showing that the first half-reaction has been completed (Fig. 5C). In subunit B the internal aldimine was observed similar to the complex found in subunit B in the 6-AHA binding study. The structures determined of PjAT resemble most the structures of OoAT with PMP bound (5GHF), of CvAT with PLP bound (PDB 4A6T) and with gabaculine-PLP bound (m-carboxyphenyl pyridoxamine phosphate) (4BA5) [26,44,45].

The results provide no indication of a major conformational change upon substrate binding since (apart from the mobility of Arg417 and Met419) the structures of PjAT with PLP, PMP, phosphate, succinate, and the external aldimine formed with 6-AHA were very similar, as also observed in the P. aeruginosa transaminase [45]. However, conformational changes upon binding of a phosphate or phosphate mimic of several loops involved in structuring the active site may be possible in ATs, like displayed in CvAT [44,48]. Both CvAT and VfAT have phenylalanine side chains pointing towards the conserved arginine (Arg417 in PjAT), and restricting space at the active site entrance (Fig. 6). A major conformational change associated with 6-AHA binding is not necessary in PjAT since Ser87, opposite to Arg417 in the active site, leaves enough space for the repositioning of Arg417 to a position where it can form a salt bridge with the carboxylate group of 6-ACA.

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Discussion

The recently identified *P. jessenii* ω-transaminase, involved in the degradation of caprolactam, was studied after expressing the enzyme in *E. coli* by measuring substrate selectivities and examining various crystal structures. The sequence and the structure indicate that the enzyme is a class III aminotransferase (or AT-II subgroup) of the fold type I superfamily of PLP enzymes. To examine if and why the enzyme shows exceptional activity with 6-AHA and 6-OHA, which are intermediates of the caprolactam degradation pathway, activities were measured with a range of substrates, and compared to those found with well-characterized ω-aminotransferases from *C. violaceum* and *V. fluvialis*. The results showed that at low concentrations 6-AHA and 6-OHA are better substrates for the new *Pj*AT as compared to these reference ω-ATs, suggesting that the enzyme has indeed evolved for efficient deamination of the 6-AHA intermediate formed by enzymatic ring opening of caprolactam. The specificity constant \(k_{cat}/K_m\) found with 6-AHA was 2-20 fold better for the *P. jessenii* enzyme, mainly due to the much higher \(K_m\) of *Vf*AT and *Cv*AT. The same is found for the aldehyde 6-OHA: the \(k_{cat}/K_m\) is at least 25-fold better in case of *Pj*AT than with the other ATs and the \(k_{cat}/K_m\) with 6-OHA is also at least 40-fold better than that with all the other tested amine substrates. In vivo levels of intermediates formed during caprolactam metabolism are unknown and may vary, but at high concentrations the aldehyde may be inhibitory for all three enzymes, as indicated by substrate inhibition. Substrate inhibition by the ketone or aldehyde acceptor observed in aminotransferases may be due to binding to the PLP form of the enzyme in competition with the amine donor. Similarly, substrate inhibition by the amine donor may be due to binding to the PMP form of the enzyme in competition with acceptor [40,41]. These effects make it difficult to assess the relevance of differences in kinetic constants for growth kinetics, but the structural studies provided further indications for a dedicated role for the *Pj*AT enzyme in 6-AHA metabolism. This role is in agreement with the upregulation of *Pj*AT found in proteomics experiments by Otzen et al. [10] when comparing cells growing on caprolactam with controls growing on glucose and ammonia.

Members of the class III ATs catalyze transfer of the amino group from an ω-amino acid, β-amino acid or from non-amino acid amines to an α-keto acid acceptor, producing an α-amino acid. Yet, these enzymes have little or no activity with α-amino acids as substrates, as found here with *Pj*AT, and thus apparently prevent the reaction of α-amino groups flanking an α-carboxylate functionality with the cofactor. Several of these ω-ATs, such as γ-aminobutyrate aminotransferase [55] and ornithine aminotransferase [56], use 2-oxoglutarate as amine acceptor in the second half reaction and deploy a gateway system to prevent reactions at α-amino groups of amino acids [17]. This gateway consists of an juxtaposed pair of a glutamate and an arginine that form a salt bridge during the first half reaction, thereby avoiding a stabilizing interaction of the arginine with the carboxylate of an α-amino acid. In the second half reaction the gateway salt bridge is lost and the arginine can interact with the α-carboxylic group of the acceptor 2-oxoglutarate.

The Arg-Glu gateway pair is present at conserved positions in the sequence of class III ATs (Table S2), but the sequences (Fig. 3) and structure (Fig. 5) indicate that *Pj*AT and its close relatives (e.g. *Vf*AT, *Cv*AT, >30% identity) do not employ such a gateway system. Sequence alignments (Fig. 3) derived from a structure-based phylogenetic tree (Fig. S1, Table S2) show that *Pj*AT contains two alanines at the Arg-Glu gateway positions. Accordingly, *Pj*AT has a preference for pyruvate over 2-oxoglutarate as amine acceptor. Instead of the gateway arginine in the last β-strand (Arg412 in

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ornithine aminotransferase, PDB 1OAT/2CAN) \(P_j\)AT possesses an arginine (Arg417, which interacts with carboxylate of 6-AHA) on a stretch of sequence (a short β-strand in \(P_j\)AT) that is more outward and connects the preceeding α-helix to the last β-strand. The external aldime of \(P_j\)AT with pyruvate (alanine) bound can be modeled by superimposing Arthrobacter AT with Ala bound (PDB 5G2Q) on \(P_j\)AT with 6-ACA bound. The rotamer of Arg417 (Arg442 in 5G2Q) is altered so that it stabilizes the external aldime by interacting with the carboxylate group of Ala. This movement of Arg417 can be regarded as a switch which moves the guanidine group of Arg417 from a position where it interacts with the carboxylate of 6-ACA in the first half reaction to a position where it interacts with the carboxylate of the alanine formed in the second half reaction. The hydrophobic sidechain of Arg417 is sandwiched between Met54 and Met419 and the Nε of Trp58 could also help in stabilizing the alanine carboxylate. Furthermore, \(P_j\)AT can convert no α-amino acids, except for a very low activity with an α-amino acid with a very small side group such as serine. This can be due to the occupation by the switching arginine of the tunnel leading to the active site, also called the O-pocket because it is in the vicinity of the O3′ atom of PLP (60,17). Arginine 417 could move in, to bind a carboxylate group, but this would leave insufficient space for larger α-amino acid side chains. A spacious pocket, like the P-pocket present in other class III ATs for binding bulky side chains, is also lacking. Thus, in comparison to Orn-AT \(P_j\)AT has a different mechanism for rejecting α-amino acids and ketoglutarate as substrates. \(P_j\)AT is also different from the 6-AHA-deamining aminotransferase proposed by Takehara et al. [9] to be involved in 6-AHA degradation by Arthrobacter sp. K172. There is only low similarity (27% sequence identity), and the sequence of the Arthrobacter protein suggests it does have a gateway system, like 4-aminobutyrate aminotransferase.

In \(P_j\)AT, the flexible arginine Arg417 plays an important role in the first half reaction, when it interacts with the carboxylate of 6-ACA as shown by the crystal structure of the external aldime. In ornithine δ-aminotransferase (Orn-AT, PDB 2CAN) the carboxylate of the bound substrate interacts with an arginine at a different position, i.e. Arg180, which is called the switching arginine. An arginine at the corresponding position is missing in \(P_j\)AT. In Orn-AT, the (non-reacting) α-amino group interacts with the hydroxyl group of Tyr55 [56]. In lysine ε-aminotransferase (Lys-AT, PDB 2CJD) the carboxylate interacts with Arg170 (switch) and the amine with Asn328 [58], and the N328A mutant has negligible lysine ε-aminotransferase activity [59]. Interestingly, the sidechains Tyr55 of Orn-AT and Asn328 of Lys-AT are at similar positions in the active sites. In contrast, \(P_j\)AT has a Gly at that position in the structure and thus lacks the specific contacts (hydrogen bonds) necessary to stabilize the α-amino of L-lysine or L-ornithine, explaining our observation that these substrates are not accepted for ε- or δ-deamination. Positioning of L-lysine in the active site for ε-deamination would introduce a positive charge close to the iminium of Arg417.

\(P_j\)AT also does not convert β-amino acids, in contrast to β-phenylalanine aminotransferases from Variovorax paradoxus (PDB 4AOA) and Mesorhizobium (PDB 2YKX [60]. Both of these enzymes enzymes can also accept α-amino acids. In these dual substrate specificity enzymes, the carboxylate groups of β-amino acids are stabilized by Arg41/54 and a backbone amide of Gly299/313, and those of α-amino acids by Arg398/412. Arg417 in \(P_j\)AT is shifted only 1 residue from Arg398/412. However, \(P_j\)AT has no stabilization for β-amino acids, a carboxylate at that position in the substrate would collide with the sidechain of Phe86, and due to the absence of a spacious P pocket the active site is too narrow for accommodating bulky side chains.

The well-studied mitochondrial Asp-AT belongs to the class I group of aminotransferases of the PLP fold-type I enzymes [17]. Although their sequences only have 9% sequence identity the structures of \(P_j\)AT and Asp-AT are similar (RMSD of 3.5 Å on 280 aligned residues) (Fig. S2). However,
PJAT does not show the closure of the active site upon substrate binding that is well established for Asp-AT [61]. The mode of substrate binding is also quite different. Asp-AT has 2 arginines for stabilization of the substrates: Arg386 for the α-carboxylic group and Arg292 for the β-carboxylic group of the aspartate. Both are absent at the corresponding topological positions on strand/loop in PJAT.

Comparison of the crystal structures of the related Class III enzymes PJAT, CvAT and VfAT and the recently solved structure of O. anthropl ω-aminotransferase (OaAT) showed that both the overall structures and the active sites are well conserved. Subtle differences can be observed in the Gly166-Asn167 (PJAT) loop which approaches the active site in PJAT and OaAT whereas in the corresponding regions of CvAT (Tyr168-Me169) and VfAT (Tyr165-Asn166) the backbone structure is further outward (Fig. 6). The distance of the Tyr-OH to the flexible arginine is about 7 Å in VfAT and CvAT, while the shortest distance from Asn167 to the 6-AHA substrate in PJAT is 6.5 Å. These distances are too long for any interaction. Another difference is the presence of Tyr20 in PJAT and OaAT, which is hydrogen bonded to the Asn in the Gly166-Asn167 loop. There is a Phe at this position in the other two ATs, making the subsite in PJAT smaller and slightly more polar. Further comparison of the active sites suggest a cause of the better recognition of 6-AHA by PJAT. In VfAT, CvAT and other ω-aminotransferases a Phe is conserved at position 87, whereas a Ser is present in PJAT and the more similar OaAT. The smaller Ser facilitates an outward motion of Arg417 that leads to a salt bridge with the terminal carboxylate of 6-AHA, rather than a collision. In line with this finding, previous studies using VfAT revealed that its substrate scope can be altered by mutagenesis of the active site. Cho et al. [49] demonstrated that the substitution of the conserved Trp57 and 147 with a Gly residue enabled VfAT to accept longer aliphatic chain substrates, and Arg415 is replaced in several protein engineering studies [46,50-54]. The creation of space that allows outward movement of Arg417 by the Phe to Ser substitution at position 87 in PJAT, which in turn makes space for the 6-ACA carboxylate, may be one of the adaptations of the enzyme to a role in caprolactam metabolism.

Substrate scope analyses and kinetic studies have shown a preference of the three aminotransferases examined here towards aromatic substrates. This is in line with previous studies using CvAT and VfAT [24,42]. Interestingly, PJAT revealed lower catalytic efficiency towards the linear substrate 1-aminopentane in comparison to 4-aminobutanoic acid. The carboxylate group in the latter substrate will also be recognized by the flexible Arg417 [53], allowing better substrate binding. In contrast, VfAT and CvAT showed the best activity with 1-aminopentane in comparison with the other linear amines tested. As mentioned, in PJAT residue Ser87 replaces a phenylalanine in CvAT and VfAT, therefore Arg417 is in a more polar environment and the combined effect may cause binding of the apolar 1-aminopentane to be unfavorable in PJAT.

In conclusion, we showed that PJAT has a non-polar active site that is shaped mainly by aromatic side chains, such as Tyr and Phe. The high sequence similarity and the conserved secondary structure confirm the identity of the enzyme as an ω-transaminase. The high catalytic efficiency and the low Km of PJAT with 6-AHA and 6-OHA in combination with the residue configuration in the active site explain the role of PJAT in the caprolactam/nylon 6 degradation pathway.
Materials and Methods

Substrates and chemicals. Nicotinamide adenine dinucleotide (NAD) and alanine dehydrogenase from Bacillus cereus (BcAlaDH) were purchased from Sigma-Aldrich. Pyridoxal phosphate (PLP) was purchased from Fisher Scientific. Potassium phosphate dibasic trihydrate and potassium phosphate monobasic were obtained from Merck. The substrates 6-aminohexanoate, 5-aminopentanoic acid, 1-aminopentane, 4-aminobutanoic acid, L-lysine, glycine, (S)-(--)α-methylbenzylamine, benzylamine, 2-phenylethylamine, 4-phenylbutylamine, 3-phenylpropylamine were purchased from Sigma-Aldrich. We synthesized 6-oxohexanoic acid as described by Bouet et al. [63].

Enzyme expression and purification. The isolation and characterization of P. jessenii strain GO3 was recently described by Otzen et al. [10]. Its 6-aminohexanoate aminotransferase was produced in E. coli strain Top10 using the PJAT gene cloned in frame downstream of the hexahistidine tag sequence in expression vector pET20b(+). The coding sequence was obtained by PCR amplification of genomic DNA extracted from strain GO3. Derivatives of the expression vector pET28b(+) containing an in-frame fusion of the C. violaceum aminotransferase (CvAT, NP_901695) or the V. fluvialis aminotransferase (VFAT, AEA9183) were described by Palacio et al. [34]. The ω-aminotransferases PJAT, CvAT and VFAT were expressed in E. coli strain C41 and purified to homogeneity using His-tag metal affinity chromatography followed by a desalting step, as described earlier [34].

Activity assays. The activities of PJAT, CvAT and VFAT coupled to pyruvate amination were measured using an indirect assay by following alanine-dependent reduction of NAD⁺ by alanine dehydrogenase at 340 nm (ε NAD⁺ = 6.22×10³ M⁻¹ cm⁻¹) [64]. Excess alanine dehydrogenase from B. cereus (BcAlaDH) was added to the reaction mixtures to ensure that the rate-limiting step is the transamination reaction. The pyruvate concentration was optimized to ensure high BcAlaDH activities and a minimal lag phase. The reaction mixtures contained variable concentrations of substrate, 2 mM NAD⁺, 0.05 mM PLP, 5 U/ml BcAlaDH, 0.015 mg/ml aminotransferase and 0.2 mM pyruvate in 100 mM potassium phosphate, pH 8. Assays were performed in flat-bottomed 96-well microtiter plates. The absorbance at 340 nm was monitored during 30 min at 30°C using a microtiter plate reader (Synergy Mx Microplate Reader, BioTek Instrument, Winooski, USA). The plates were pre-warmed and reactions were started by addition of 150 µl of 0.4 mM pyruvate solution to 150 µl reaction mixture. All assays were done in triplicate. The initial rates of the reduction of NAD⁺ observed with different substrate concentrations were used to determine the kinetic constants. Specific activities are expressed in units per mg of protein (µmole·min⁻¹·mg⁻¹).

Amination of 6-oxohexanoic acid was also measured using the coupled assay, i.e. by following the pyruvate-dependent oxidation of NADH by alanine dehydrogenase. The reaction mixtures contained 100 mM potassium phosphate buffer (pH 8), 2 mM substrate, 0.1 mM NADH, 0.05 mM PLP, 8 U/ml BcAlaDH, 5 mM ammonium bicarbonate, 5 mM L-alanine and varying concentrations of substrate in a total volume of 300 µl. Reactions were initiated by addition of L-alanine and carried out as described above.

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**Crystallization and X-ray data collection.** The protein was concentrated to 10.5 mg·ml⁻¹ in 25 mM HEPES buffer pH 7.5 using a Vivapsin Turbo 4 30K filter unit (Sartorius). Initial vapour diffusion crystallization experiments were performed using a Mosquito crystallization robot (Molecular Dimensions Ltd, Newmarket, England). Various commercially available crystallization screens were used, e.g. JCSG+ and PACT (Qiagen Systems, Maryland, USA) and Cryo (Emerald Biosystems, Bainbridge Island, USA). *PjAT* crystals were obtained at room temperature from succinate and ammonium phosphate (both pH ~7.5) solutions. Optimization of the crystallization conditions yielded well-diffracting colourless crystals that grew within a few days when 1 µl protein solution (7.5 mg·ml⁻¹) was mixed with 1 µl reservoir solution containing 0.7-1.0 M ammonium phosphate or succinate, pH 7.3. Yellow PLP-containing protein crystals could be grown by first incubating *PjAT* with 0.1 mM fresh PLP for a few hours and using succinate as precipitant.

Before data collection, crystals were briefly soaked in a cryoprotectant solution consisting of 30% glycerol and 1.2 M succinate, pH 7.5, in 30% glycerol and 1.2 M ammonium phosphate, pH 7.5, or in 30% glycerol, 1.2 M succinate and 0.1 mM PLP. The ligand complexes were obtained by soaking a PLP-containing crystal in succinate solution containing 0.4 M 6-AHA or (S)-(−)-α-methylbenzylamine ((S)-MBA) for 30 s and then briefly soaked in cryoprotectant including 6-AHA or (S)-MBA. X-ray diffraction data were collected with an in-house MarDTB Goniostat system using Cu-Kα radiation from a Bruker MicrostarH rotating-anode generator equipped with HeliosMX mirrors at 100 K. Intensity data were processed using XDS [65] and the CCP4 package [66]. The space group was P4₃, with unit cell dimensions of a = b = 98.4 Å and c = 119.3 Å. With two *PjAT* monomers of 50 kDa in the asymmetric unit, the Vₘ is 2.9 Å³/Da [67] with a calculated solvent content of 57%. A summary of data collection statistics is given in Table S1.

Using the FFA503 server [68] and SCWRL [69], a homology model for *PjAT* was generated. Molecular replacement was performed with PHASER [70]. Phenix Phase and Build [71] was used for automatic building and COOT [72] was used for manual rebuilding and map inspection. The model was refined with REFMAC5 [73] with local NCS restraints and with TLS rigid body refinement as the last step, resulting in a final model comprising 2 protein molecules forming a homodimer. No significant conformational changes are observed between native and ligand bound enzymes. The Ca atoms of all five models superimpose with root mean square deviations of 0.1-0.2 Å. The quality of the models was analyzed with MolProbity [74]. Figures were prepared with PyMOL [75] and ESPript [76].

**Phylogenetic analyses.** The sequences of several transaminases present in the PDB database were structurally aligned using the ProMals3D webserver (http://prodata.swmed.edu/promals3d). The multiple sequence alignment was provided to the MEGA X software to perform evolutionary analyses with default settings [62].

**Accession numbers.** Atomic coordinates and experimental structure factor amplitudes are accessible at the protein databank (https://www.rcsb.org/) as entries 6G4B (E·succinate complex), 6G4C (E·phosphate complex), 6G4D (E·PLP complex), 6G4E (E·PLP-6-aminohexanoate complex), and 6G4F (E·PMP complex).

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Authors contribution

All authors designed experiments and contributed to the interpretation of the data. C.M.P. cloned and isolated the enzyme. C.M.P. and Q.M. performed kinetic experiments and H.R. solved the crystal structures. C.M.P., H.R., M.O. and D.B.J. wrote the manuscript.

Acknowledgments

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Supporting Information

Table S1: Crystallographic data collection and refinement statistics. Table S2: Class II/III TAs used for phylogenetic analysis and groups interacting with substrate. Fig. S1: Phylogenetic analysis of transaminases. Figure S2: Crystal structure of PjAT and aspartate aminotransferase.

References


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<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>$Pj\text{AT}$</th>
<th>$Cv\text{AT}$</th>
<th>$Vf\text{AT}$</th>
</tr>
</thead>
<tbody>
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<td>100</td>
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<td>-</td>
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<td>-</td>
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<tr>
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<td>60</td>
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<tr>
<td>4-Phenylbutylamine</td>
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<td>145</td>
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<tr>
<td>3-Fluorobenzylamine</td>
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<td>75.5</td>
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<td>53.6</td>
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<td>4-Fluorobenzylamine</td>
<td><img src="image16" alt="Structure" /></td>
<td>67.2</td>
<td>94.8</td>
<td>78.7</td>
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<td>L-Serine</td>
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<td>3.1</td>
<td>10.7</td>
<td>3.8</td>
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<td>L-Leucine</td>
<td><img src="image18" alt="Structure" /></td>
<td>&lt;1</td>
<td>4.7</td>
<td>18.6</td>
</tr>
</tbody>
</table>

*a* Reaction conditions: 15 mM of substrate, 0.2 mM pyruvate, 0.05 mM PLP, 5 U/ml alanine dehydrogenase, 2 mM NAD, and 0.015 mg/ml CvAT, VfAT or PjAT, 30°C, in 100 mM potassium phosphate, pH 8. Substrates for which no or very low activity was detected with all three enzymes include 2-(2-butoxyethoxy)ethanamine, 2-aminooctanoic acid, 1-butoxy-2-propanamine, (S)-3-amino-3-phenylpropanoic acid ethyl ester, (S)-3-amino-3-(p-hydroxyphenyl)proionic acid, L-serine, L-tyrosine, L-phenylalanine, L-ornithine, L-valine, L-aspartate, L-glutamate, L-tryptophan, L-alanine, β-(R)-phenylalanine, and β-(R)-tyrosine. *PjAT* also showed no activity with L-and D-lysine, D-phenylalanine, D-ornithine, L- and D-glutamic acid, ethylamine, 1,3-diaminopropane, 1,4-diaminobutane, spermidine

*b* Activities are expressed as % of the activity found with (S)-α-methylbenzylamine. Values in parenthesis represent specific activities with (S)-α-methylbenzylamine (U/mg).

*c* Not determined.
Table 2. Steady state kinetic parameters of the aminotransferases for linear aliphatic and aromatic substrates.\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>(k_{\text{cat}}) (s(^{-1}))(^b)</th>
<th>(K_M) (mM)</th>
<th>(K_i) (mM)</th>
<th>(k_{\text{cat}}/K_M) (mM(^{-1}).s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Caprolactam metabolism intermediates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Aminohexanoate</td>
<td>(Pj)AT</td>
<td>0.3 ± 0.1</td>
<td>1.54 ± 0.1</td>
<td>1.54 ± 0.1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>(Cv)AT</td>
<td>0.95 ± 0.02</td>
<td>10.6 ± 0.4</td>
<td>10.6 ± 0.4</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>(Vf)AT</td>
<td>0.58 ± 0.01</td>
<td>71 ± 2</td>
<td>71 ± 2</td>
<td>0.008</td>
</tr>
<tr>
<td>6-Oxohexanoate(^b)</td>
<td>(Pj)AT</td>
<td>5.4 ± 0.1</td>
<td>0.06 ± 0.01</td>
<td>1.26 ± 0.6</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>(Cv)AT</td>
<td>11 ± 2</td>
<td>2.8 ± 0.9</td>
<td>3.3 ± 0.9</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>(Vf)AT</td>
<td>1.8 ± 0.1</td>
<td>2 ± 0.2</td>
<td>11 ± 1</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>Linear aminated substrates</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Aminopentanoic acid</td>
<td>(Pj)AT</td>
<td>0.21 ± 0.01</td>
<td>0.9 ± 0.1</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>(Cv)AT</td>
<td>0.35 ± 0.01</td>
<td>9.8 ± 0.4</td>
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<td>0.04</td>
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<tr>
<td></td>
<td>(Vf)AT</td>
<td>0.02 ± 0.01</td>
<td>8.2 ± 1.3</td>
<td></td>
<td>0.003</td>
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<tr>
<td>4-Aminobutanoic acid</td>
<td>(Pj)AT</td>
<td>0.26 ± 0.01</td>
<td>2.1 ± 0.1</td>
<td></td>
<td>0.1</td>
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<tr>
<td></td>
<td>(Cv)AT</td>
<td>0.55 ± 0.01</td>
<td>2.3 ± 0.1</td>
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<tr>
<td></td>
<td>(Vf)AT</td>
<td>0.3 ± 0.1</td>
<td>8.9 ± 0.4</td>
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<td>0.03</td>
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<tr>
<td>1-Aminopentane</td>
<td>(Pj)AT</td>
<td>0.18 ± 0.01</td>
<td>8.5 ± 0.5</td>
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<td></td>
<td>(Cv)AT</td>
<td>1.1 ± 0.1</td>
<td>5.7 ± 0.5</td>
<td>117 ± 27</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>(Vf)AT</td>
<td>0.6 ± 0.1</td>
<td>7.2 ± 0.7</td>
<td>64 ± 13</td>
<td>0.08</td>
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<td><strong>Aromatic aminated substrates</strong></td>
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<td></td>
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<tr>
<td>Benzylamine</td>
<td>(Pj)AT</td>
<td>0.75 ± 0.04</td>
<td>1.8 ± 0.2</td>
<td>&gt;30</td>
<td>0.4</td>
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<tr>
<td></td>
<td>(Cv)AT</td>
<td>≥1.6 ± 0.1(^d)</td>
<td>≥1.0 ± 0.1(^d)</td>
<td>18 ± 2(^d)</td>
<td>1.6</td>
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<tr>
<td></td>
<td>(Vf)AT</td>
<td>0.74 ± 0.02</td>
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<td>&gt;200</td>
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<tr>
<td>(S)-(α)-Methylbenzylamine</td>
<td>(Pj)AT</td>
<td>1.1 ± 0.1</td>
<td>2.1 ± 0.2</td>
<td>22 ± 2</td>
<td>0.5</td>
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<tr>
<td></td>
<td>(Cv)AT</td>
<td>2.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>59 ± 7</td>
<td>1.7</td>
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<tr>
<td></td>
<td>(Vf)AT</td>
<td>1.6 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>151 ± 38</td>
<td>1.5</td>
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<td>0.75 ± 0.04</td>
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<td>5 ± 0.1</td>
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<tr>
<td></td>
<td>(Vf)AT</td>
<td>0.3 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>3-Phenylpropylamine</td>
<td>(Pj)AT</td>
<td>0.13 ± 0.01</td>
<td>2.4 ± 0.2</td>
<td>44 ± 6</td>
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<tr>
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<td>(Cv)AT</td>
<td>0.38 ± 0.01</td>
<td>1.7 ± 0.1</td>
<td>46 ± 4</td>
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<tr>
<td></td>
<td>(Vf)AT</td>
<td>0.21 ± 0.01</td>
<td>0.77 ± 0.04</td>
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<td>0.3</td>
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<tr>
<td>4-Phenylbutylamine</td>
<td>(Pj)AT</td>
<td>0.3 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>&gt;200</td>
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<tr>
<td></td>
<td>(Cv)AT</td>
<td>0.43 ± 0.01</td>
<td>0.56 ± 0.04</td>
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<tr>
<td></td>
<td>(Vf)AT</td>
<td>0.1 ± 0.01</td>
<td>0.71 ± 0.05</td>
<td></td>
<td>0.1</td>
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</tbody>
</table>

\(^a\) Reactions with amine donors were carried out in triplicate with varying substrate concentrations (0.1-90 mM), 0.2 mM pyruvate, 0.05 mM PLP, 5 U/ml alanine dehydrogenase, and 0.015 mg/ml \(Cv\)AT, \(Vf\)AT or \(Pj\)AT at 30°C in 100 mM potassium phosphate, pH 8.

\(^b\) Values for \(k_{\text{cat}}\) are per subunit.

\(^c\) Substrate amination reactions were carried out in triplicate with 0.08-32 mM 6-oxohexanoate, 0.05 mM PLP, 0.1 mM NADH, 5 mM L-alanine, 5 mM ammonium bicarbonate, 8 U/ml \(Bc\)AlaDH, and 100 mM potassium phosphate buffer, pH 8, at 30°C.

\(^d\) Only lower limits for \(k_{\text{cat}}\) and \(K_m\) were obtained due to complex substrate inhibition.
Figure 1. Nylon 6, nylon 6-oligomer and caprolactam biodegradation. The catabolic pathways of 6-aminohexanoate (cyclic) dimers and oligomers [11,12] and the route of caprolactam degradation in *P. jessenii* [10] converge to 6-aminohexanoate, which is deaminated in *P. jessenii* by the aminotransferase described here.
**Figure 2. Crystal structure of *P. jessenii* aminotransferase.** One subunit is colored in red/blue/grey and the other in green. The external aldimines of PLP bound to 6-aminohexanoate in both subunits are shown as yellow spheres. Residues from both subunits contribute to each active site.
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Figure 3. Structure–based sequence alignment of eight PLP fold type I class III aminotransferases. Displayed are the sequences of PjAT from *P. jessenii*, OaAT from *Ochrobactrum anthropi* (5GHF, [26]), a putative AT from *Mesorhizobium loti* maff303099 (MIAT, 3GJU, JCSG), ω-AT from *Paracoccus denitrificans* (PdAT, 4GRX, [25]), VfAT from *V. fluvialis* (4E3Q, [46]), AT from *Silicibacter pomeroyi* (SpAT, 3HMU, NYSGXRC), a putative AT from *Silicibacter* sp. TM1040 (SiAT, 3FCR, JCSG), and CvAT from *C. violaceum* (4A6R, 4A6T, 4BA5 [44,45,48]). The sequences have more than 40% identity. The secondary structure elements above the sequence alignment are obtained from the crystal structure of PjAT. Identical residues have a red background color and similar residues have a red color. Residues with a purple color are the catalytic lysine and with a green color line the active site, the cyan asterisks below the sequences indicate the position of the gateway Arg and Glu residues which are missing in the displayed sequences. The figure was created with ESPript [49].
Figure 4. Active site structure of PjAT. A) Binding of a phosphate ion (in orange) in ammonium phosphate-grown crystals. B) Binding of a succinate molecule (in orange) in the phosphate binding site. The glycerol molecule (in pink) occupies a small pocket. The residues forming the active site are displayed as sticks in cpk colors, Lys287 in green and Thr324’ from the other subunit in dark green. Waters are depicted as red spheres. Hydrogen bonds are shown as dotted lines with indicated distances in Å. Ser292 shows a double conformation.
Figure 5. Structures of the active site of PjAT with PLP in different forms. A) Internal aldime with PLP (yellow) bound to the ε-nitrogen of Lys287 (light green) as a Schiff base. B) Structure of the 6-AHA–PLP (yellow-magenta) external aldimine adduct, with Arg417 forming a bidentate salt bridge to the carboxylate of 6-AHA. Residue Met419 presents a double conformation. C) PLP in the pyridoxamine (PMP) form after reaction with (S)-MBA. Residues forming the active site are displayed as sticks in cpk colors, Lys287 in green, Phe86' and Ser87' from the other subunit in dark green and the glycerol molecule in cyan. Black dotted lines with distances in Å indicate hydrogen bonds.
Figure 6. Overlay of the active sites of PLP-bound forms of *Pj*AT, *Oa*AT, *Cv*AT and *Vf*AT in stereo view. *Pj*AT in gray, with internal aldimine complex in yellow, *Oa*AT PMP bound form (PDB code 5GHF) in green, *Cv*AT PLP bound form (4AH3) in cyan (with R416 in double conformation) and *Vf*AT in the PMP bound form (4E3Q) in magenta. Only residues that differ between the three enzymes and the flexible arginine are shown.