Chapter 4

Crystallographic snapshots along the reaction pathway of the (R)-selective ω-transaminase ArR-ωTA from *Arthrobacter* sp. KNK168

Eswar Reddy Reddem, Niels van Oosterwijk, James L. Galman, Nicholas J. Turner, Bauke W. Dijkstra, and Andy-Mark W.H. Thunnissen*

The gene encoding the (R)-selective ω-transaminase from *Arthrobacter* sp. has been expressed in *E. coli*, and the protein was purified and crystallized. Crystals were briefly soaked with various substrates and inhibitors to give insights into the enzyme’s enantioselectivity and substrate specificity.

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Abstract

The (R)-selective \( \omega \)-transaminase from \textit{Arthrobacter} sp. KKN168 (ArR-\( \omega \)TA) is able to convert a wide range of ketones to industrially important chiral amines, using D-alanine as an amino donor. Here, we present four crystal structures of ArR-\( \omega \)TA: two with the pyridoxal 5-phosphate cofactor (PLP) in the internal aldimine form, one with PLP in the aminated form, and one with PLP in an intermediate state resulting from a short soak of catalytically active crystals with D-alanine. In addition, two crystal structures are presented of ArR-\( \omega \)TA treated with the suicide inhibitors gabaculine and D-cycloserine. The ArR-\( \omega \)TA protein is a homodimer with a molecular weight of 75 kDa and belongs to fold class IV of PLP-dependent enzymes. Each monomer has its active site at the dimer interface with amino acid residue contributions from both subunits. A highly conserved lysine residue in the active site, Lys188, forms an Schiff base with PLP in the internal aldimine form. The structure of the intermediate state reveals the geometry of the PLP-D-Ala aldime (external aldimine) with the \( \alpha \)-carboxyl and \( \alpha \)-methyl groups occupying two pockets of different size. The Ca-H proton points towards the side chain amino group of Lys188, consistent with a role of this residue as catalytic acid/base during the conversion of the external aldimine to the ketimine intermediate. Arg138 in a highly flexible active-site loop participates in binding of the PLP-D-Ala intermediate by electrostatic interactions with the D-Ala \( \alpha \)-carboxylate. The ArR-\( \omega \)TA structures reveal the determinants for enantioselectivity and substrate specificity that are common to other, related (R)-selective \( \omega \)-transaminases. The flexible active-site loop allows for dual substrate recognition in the larger pocket, with significant contribution of Arg138, bearing some similarity to the “arginine switch” in (S)-selective \( \omega \)-TAs.
Introduction

ω-Transaminases (ω-ATs E.C 2.6.1.X) are enzymes that catalyze the transfer of an amino group from an amino donor to a carbonyl moiety of an amino acceptor, whereby one of the two compounds can be a non-activated aldehyde, ketone or amine (Koszelewski et al., 2010). For their catalytic action these enzymes use pyridoxal 5’-phosphate (PLP, a derivative of vitamin B6) as a prosthetic group, which is covalently bound to an active site lysine residue via a Schiff base (the "internal aldimine"; Meister et al., 1954). PLP-dependent enzymes have been classified into seven different fold types (Jansonius, 1998; Percudani & Peracchi, 2009), with ω-ATs belonging to either fold type I or fold type IV. So far, all fold type I ω-ATs are (S)-selective enzymes, capable of deaminating (S)-α-methylbenzylamine ((S)-α-MBA) (Kaulmann et al., 2007; Höhne & Bornscheuer, 2009; Truppo et al., 2009; Mutti et al., 2011; Iwasaki et al., 2012; Park et al., 2012; Kroutil et al., 2013; Midelfort et al., 2013). In contrast, fold type IV ω-ATs catalyze the transamination of (R)-methylbenzylamine ((R)-α-MBA) (Höhne et al., 2010; Savile et al., 2010; Malik et al., 2012; Park & Shin, 2013). The (R)-selective ω-ATs are structurally and functionally related to the well-studied D-amino acid aminotransferase (d-AAT) and branched-chain amino acid transaminase (BCAT) from *E. coli* (Sugio et al., 1995; Goto et al., 2003).

The transfer of an amino group by an ω-AT is a reversible reaction, which is accomplished via two half reactions (Figure 1). The first half reaction is the oxidative deamination of an amino donor with concomitant conversion of PLP to PMP.
Figure 1: General amino-transfer reaction catalysed by an (R)-selective \( \omega \)-TA. (A) Reversible reaction scheme, showing the two half cycles by which an amino group is transferred from an amino donor (e.g. D-alanine) to an amino acceptor (a ketone), producing a chiral (R)-amine, with the help of PLP and PMP. (B) Chemical structures of the PLP internal aldimine form and PMP.

(pyridoxamine 5’-phosphate). In this half reaction, the amino group of the incoming substrate reacts with the PLP, and replaces the Schiff base bond of the internal aldimine forming the external aldimine intermediate. The released free lysine can now act as a base that mediates the transfer of a proton from the Ca atom of the external aldimine to the C4’ atom of the PLP (the 1,3-prototropic shift). Hydrolysis of the resulting intermediate yields PMP. In the second half reaction a reductive amination takes place, in which the amino group of PMP is transferred to a keto-acid, ketone or aldehyde, regenerating the PLP internal aldimine and producing a new amino acid or amine. Apart from being enantioselective, the function of \( \omega \)-ATs is characterized by multiple substrate recognition, allowing binding and conversion of amino donors and acceptors with different side chains. In particular, the transfer of the amino group from an \( \alpha \)-amino acid to a ketone, producing a chiral amine with conservation of the stereochemistry at the Ca-atom, requires the ability to bind either a charged \( \alpha \)-carboxylate group or a hydrophobic \( \alpha \)-alkyl/aryl group at the same location of the active site (dual specificity pocket). To accomplish this dual recognition, (S)-selective \( \omega \)-ATs utilize a so-called “arginine switch”, common to many fold-type I transaminases (Hirotu \textit{et al.}, 2005), \textit{i.e.}, an active-site arginine residue which shifts its position to either electrostatically interact with the carboxylate group or to move aside to allow access of an aliphatic/aromatic group. It is not clear whether (R)-selective \( \omega \)-ATs utilize a similar mechanism. The related enzyme d-AAT from \textit{E. coli} makes use of an arginine (Arg98*) in a loop of an adjacent subunit in the dimer (the so called “active-site loop”) to provide electrostatic stabilization of the carboxylate group during formation of external aldimine state (Sugio \textit{et al.}, 1995). The sequence of this loop is highly variable in (R)-selective \( \omega \)-ATs and Arg98 of d-AAT is not conserved.

\( \omega \)-ATs can be applied for the production of enantiopure compounds (Koszelewski \textit{et al.}, 2010; Mangion \textit{et al.}, 2012; Mathew & Yun, 2012; Kohls \textit{et al.}, 2014). Ideally, both (S)-selective and (R)-selective transaminases should be available. Until now (S)-selective \( \omega \)-ATs have been
Chapter 4  Crystal structure of ArR-ωTA

characterized most extensively, both structurally and functionally (Park et al., 2010; Midelfort et al., 2013; Rausch et al., 2013; Łyskowski et al., 2014; Sayer et al., 2014; Thomsen et al., 2014). In 2003, a highly (R)-selective ω-transaminase was isolated from the soil microorganism Arthrobacter sp. KKN168 (Iwasaki et al., 2003), which had a broad substrate range (Truppo et al., 2009; Iwasaki et al., 2012), making it suitable for the production of many different (R)-enantiomeric amines (Truppo et al., 2009; Mutti et al., 2011; Kroutil et al., 2013; Sehl et al., 2013). A major success with this enzyme was published by Savile et al. (2010) who successfully applied protein engineering to make the enzyme suitable for production of the drug sitagliptin, which is used by people with type-2 diabetes to control high blood sugar levels. The same, engineered enzyme was also used to produce the suvorexant drug, which is used to treat insomnia (Mangion et al., 2012; Kohls et al., 2014). Other (R)-selective ω-ATs have been identified by genomic database mining, based on the presence of conserved sequence motifs, but only a few are characterized biochemically and until recently no crystal structures of these enzymes were available. In 2014, the first crystal structures have been published of three related (R)-selective ω-ATs from the fungi Aspergillus fumigatus, Aspergillus terreus and Nectria haematococca (Łyskowski et al., 2014; Sayer et al., 2014; Thomsen et al., 2014) with bound PLP as an internal aldimine, or with a PLP derivative resulting from reaction with the suicide inhibitor gabaculine. The structures of the fungal (R)-selective ω-ATs allowed identification of the key active site residues associated with co-factor binding, enantioselective substrate binding and catalysis. A full determination of the catalytic mechanism and multi-substrate specificity of these enzymes requires determination of additional structures in different functional states, in particular of the PLP-external aldimine intermediate and PMP-bound state.

In this paper we describe a comprehensive X-ray crystallographic analysis of ArR-ωTA (also known as ATA117), the (R)-selective transaminase from Arthrobacter sp. KKN168. Using catalytically active crystals, we were able to obtain structures of this enzyme in the internal aldimine state, the PMP-bound state, and an external aldimine-like intermediate state formed upon reaction with D-Ala. In addition, crystal structures were determined of ArR-ωTA after reaction with the suicide inhibitors gabaculine and D-cycloserine. The structures provide detailed insights into the key protein–substrate interactions, revealing common determinants for enantioselectivity and substrate specificity as those previously established for the fungal
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(R)-selective ω-TAs. In addition, a mechanism for dual substrate recognition is discussed for ArR-ωTA akin to the “arginine switch” in (S)-selective ω-TAs.

Materials and methods

Expression and purification — ArR-ωTA was produced by overexpression in E. coli Rosetta II (DE3) cells (Novagen) using a pET-21a DNA plasmid containing the relevant gene (Mutti et al., 2011). The cells were grown in LB media supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. An overnight pre-culture grown at 310 K was used to inoculate the main production culture (supplemented with 0.1 mM vitamin B6), which was grown at 310 K until an OD₆₀₀ of 0.8. Subsequently, expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside, and the cells were incubated for an additional 3 h at 310 K. The cells were harvested by centrifugation at 8000g for 15 min, resuspended in 50 mM Tris-HCl buffer, pH 8.0, 300 mM NaCl and 50 µM PLP containing protease inhibitors (Complete Mini EDTA-free, Roche Applied Science) and a spatula tip of DNase I was added before disrupting the cells using a French press (Thermo ICE). The resulting material was centrifuged for 45 min at 36000g and the supernatant was passed through a 0.22 µM filter (Millipore) to remove traces of unlysed cells and aggregates. For purification, the clarified cell lysate was applied to a gravity flow column containing 5 mL Ni-NTA (Qiagen). Bound ArR-ωTA eluted from the column at approximately 125-150 mM imidazole. The sample obtained after Ni-NTA purification was diluted 6-fold with buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 µM PLP) and loaded on a 6 ml ResourceQ column (GE Healthcare). The protein was eluted from the column by a linear gradient ranging from 50 mM to 1 M of NaCl in buffer A. The fractions containing ArR-ωTA were pooled and concentrated to 50 mg/ml using a concentrator with a molecular-weight cut-off of 10 kDa (Millipore). Next, the sample was injected onto a Superdex 200 10/300 GL (GE Healthcare) column, equilibrated with 50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 50 µM PLP. The resulting fractions containing ArR-ωTA were pooled and concentrated to 30 mg/ml, flash frozen in liquid nitrogen and stored at -80°C. Protein purity and homogeneity were assessed by means of SDS-PAGE and dynamic light scattering (DynaPro NanoStar; Wyatt Technology), respectively. Typically, 20 mg of pure protein was obtained from 1 L culture. Purified protein was either used immediately for crystallization screening or stored at -80°C.
**Crystallization of the internal aldime state** — Various commercially available crystallization screens, e.g. JSCG* (Qiagen), Pact premier, Structure, Cryo (Molecular Dimensions), Wizard Screen (Emerald Biosystems), were used to screen for initial crystallization conditions using sitting drop MRC 96-well crystallization plates. Crystallization drops were set up by mixing 200 nL protein solution (30 mg/ml) with an equal volume of precipitant reservoir solution at 289 K using a Mosquito crystallization robot (TTP Labtech). Two crystallization lead conditions were found, resulting in the growth of differently shaped crystals. Plate-like crystals (orthorhombic lattice, average dimensions 100×20×100 μm³) were grown in 1 M sodium citrate and 0.1 M imidazole, pH 8.0, while rod-shaped crystals (tetragonal lattice, average dimensions 300×50×400 μm³) were obtained from 0.1 M sodium propionate/sodium cacodylate/Bis-Tris propane (PGBT) buffer, pH 6.0, and 25% PEG 1500. Both crystal types were yellow-colored, indicative of the presence of bound PLP (later identified to be covalently linked to the protein as the internal aldime). Unfortunately, the orthorhombic crystals could not be reproduced, while the tetragonal crystals were easily reproduced manually by vapour-diffusion hanging-drop experiments (drops containing 1 μL protein and 1 μL reservoir solution).

**Trapping PMP-bound, external aldime and inhibited states** — To trap relevant functional states of the protein, rod-shaped tetragonal crystals were soaked for short, varying times (10-60 seconds) with D-alanine, gabaculine (3-amino-2,3-dihydrobenzoic acid; GABA) or D-cycloserine (DCS) at varying concentrations (10-200 mM) in mother liquor (0.1 M PCTP buffer (a mixture of sodium propionate, sodium cacodylate and Bis-Tris propane in a 2:1:2 molar ratio), pH 6.0, and 25% PEG 1500), supplemented with 20% PEG 400. After soaking, the crystals were immediately flash-cooled in the cold (110K) nitrogen gas stream of the X-ray camera’s cryostat and used for diffraction data collection. Addition of PEG 400 was necessary for optimal cryo-protection of the soaked crystals. All soaking experiments were performed at room temperature. Soaking times and concentrations of substrates and inhibitors were optimized based on analysis of electron density maps and diffraction data statistics. PMP-bound crystals were also obtained by co-crystallization, by adding 50 mM D-alanine to the protein prior to setting up crystallization experiments at the same condition that yielded the tetragonal crystals. Similar co-crystallization and soaking experiments were performed with L-alanine (200 mM, up
to 2 h soaking time), but analysis of electron density maps, calculated with the diffraction data from the resulting crystals, revealed no change of the internal aldime state.

**Data collection and structure determination** — Diffraction data were partly collected in-house at 110 K using a Microstar rotating anode X-ray source (Bruker AXS GmbH) in combination with Helios optics (Incoatec GmbH) and a MAR345dtb detector (Marresearch GmbH). Additionally, diffraction data were collected at 100 K on beamline ID29 at the ESRF synchrotron, Grenoble, France. All diffraction images were indexed and integrated with XDS (Kabsch, 2010), while data scaling and merging was performed using SCALA (Evans, 2006) from the CCP4 software suite (Winn et al., 2011). The structures of the orthorhombic and tetragonal crystal forms of ArR-ωTA in the internal aldime state were independently determined by molecular replacement using the program Phaser (McCoy et al., 2007) with the structure of the branched-chain amino acid aminotransferase from *Thermus thermophilus* (PDB entry 1wrv, unpublished) as a search model. The resulting models from molecular replacement were corrected and extended by automatic model building using the program ARP/wARP (Langer et al., 2008). Several manual model-building cycles using Coot (Emsley et al., 2010) alternated with restrained atomic coordinate and B-factor refinement using Refmac5 (Murshudov et al., 2011), were necessary to complete the two internal aldime structures. The other structures with bound PMP, and with the PLP-substrate/inhibitor derivatives, were built using the tetragonal internal aldime structure of ArR-ωTA as input model. Model building of the PLP-adducts was guided by careful analysis of 2Fo-Fc and Fo-Fc Fourier maps. Stereoechemical restraints for the PLP-adducts were generated using the PRODRG2 server (Schüttelkopf & van Aalten, 2004) and adapted if necessary. Final refinement cycles were performed using Phenix:refine (Langer et al., 2008). After refinement, all models were validated using Molprobity (Chen et al., 2010).

**Docking studies** — Docking was performed with YASARA (Krieger et al., 2002) using a dimeric polypeptide model derived from the ArR-ωTA crystal structures. The hm_build.mcr macro of the YASARA package with default parameters was used. The PLP adducts of D-Ala, (R)-α-methylbenzylamine, (R)-α-ethylbenzylamine, and (R)-1-methyl-3-phenylpropylamine were generated in YASARA, and subjected to energy minimization to obtain the lowest energy conformation. Docking was performed with flexible ligands and a partly flexible active site. The
simulation cell containing the active site was defined to be 26 × 22 × 23 Å³ around the catalytic Lys188. Selected protein residues with flexible side chains were Leu61*, His62*, Val69, Phe122, Tyr150, Lys188 and Trp192 (* indicates that the residue is from an adjacent subunit). Docking was first performed in the tetragonal ArR-ωTA•PMP/PLP-D-Ala crystal structure, after removing all ligands and water molecules from the active site pocket. The active-site loop was not included. Subsequently, docking was repeated in the orthorhombic crystal structure, including the active-site loop. Correct docking poses for the different ligands were selected by comparing the orientation of the PLP moiety with that observed in the crystal structures.

**Site-directed mutagenesis** — Site-directed mutations were introduced into the ArR-ωTA gene cloned in the pET-21a expression vector. Synthetic oligonucleotide primers were purchased from Eurofins MWG (Ebersberg, Germany) and were designed to contain the desired mutation and to anneal to the complementary DNA strand (Table 1). The mutagenesis procedure followed using the instructions of the Thermo Scientific Phusion® Site-Directed Mutagenesis Kit, using Phusion high-fidelity DNA polymerase from New England Biolabs (Ipswich, MA, USA). The PCR thermocycler was an Eppendorf temperature gradient Mastercycler (Hauppauge, NY). The mutated plasmids were overexpressed in *E. coli* Rosetta II (DE3). All microorganisms were cultivated in Luria-Bertani (LB) medium at 37 °C with 50 μg/mL ampicillin added to the *E. coli* medium. All isolated plasmids were sequenced via Eurofins MWG to confirm desired mutations.

<table>
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<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tr>
<td>For: Y67F</td>
<td>5’- GATGTTACCTTTACCGTGTT -3’</td>
</tr>
<tr>
<td>Rev: Y67</td>
<td>5’- ACTATGCAGATAGCCTG -3’</td>
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<tr>
<td>For: D138A</td>
<td>5’- GTGAAACGTGCTATTACCA -3’</td>
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<tr>
<td>For: D138Q</td>
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<tr>
<td>Rev: D138</td>
<td>5’- CCGGTGTGCTGCTATAAC -3’</td>
</tr>
</tbody>
</table>

**D-amino acid oxidase (D-AAO) activity assay** — A stock solution of substrate and reagents was made up of the following: 4.95 mM 2,4,6-tribromo-3-hydroxybenzoic acid (TBHBA), 1.65 mM 4-aminoantipyrene, 11 mM sodium pyruvate, 6 mg mL⁻¹ HRP (Horseradish peroxidase), 10 mg/ml PLP, and 11 mM of the amine donor in MeOH was added to 50 mM Tris-HCl and adjusted to pH 8.0. The stock solution (90 μL) was dispensed to individual wells in a 96 well plate. 5 μL of D-AAO (4.5 U/ml) was added to each well and the assays were initiated upon addition of ArR-
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\( \omega \)TA (50 \( \mu \)g). Experiments were run on a spectrophotometer at 30 °C with the activity measured at a wavelength of 510 nm and the absorbance taken every 30 seconds. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce one \( \mu \)mole of \( \text{H}_2\text{O}_2 \) per min. The relative activities were calculated as a percentage (100%, R-MBA).

Results and discussion

Structure determination of ArR-\( \omega \)TA in the internal aldime state

ArR-\( \omega \)TA (carrying a C-terminal His\(_6\)-tag) was successfully expressed, purified and crystallized. The presence of PLP (50 \( \mu \)M) during all steps ensured that the protein crystallized in the PLP-bound, internal aldime state. Two different crystal forms were obtained: tetragonal crystals (space group \( P4_22_2 \)) with one molecule in the asymmetric unit, and orthorhombic crystals (space group \( P2_12_12_1 \)) with two molecules in the asymmetric unit. The crystal structure of ArR-\( \omega \)TA was elucidated by molecular replacement, and refined at 2.0 Å and 2.3 Å resolution, for the tetragonal and orthorhombic crystal form, respectively (see Table 2 for relevant crystallographic statistics). In both models absent density precluded placement of the first nine N-terminal residues of ArR-\( \omega \)TA in addition to the C-terminal polyhistidine-tag. While in the orthorhombic crystal structure the active-site loop (residues 130-144) was clearly visible in the electron density maps, in the tetragonal crystal structure this loop could not be modelled due to high disorder. Except for this active-site loop, the tetragonal and orthorhombic crystal structures of ArR-\( \omega \)TA are highly similar, with rmsd (root-mean-square-deviation) values for the C\(_a\) atom positions of \( \sim 0.4 \) Å. Tight dimers (buried dimeric interface of \( \sim 2230 \) Å\(^2\)/subunit) are present in both crystals (“formed” via crystallographic 2-fold axes in the tetragonal crystals). This observation is consistent with gel filtration and dynamic light scattering analysis showing that ArR-\( \omega \)TA forms 75 kDa dimers in solution (data not shown). Unfortunately, the orthorhombic crystals could not be reproduced; subsequent crystallization experiments were only successful with the tetragonal crystal form.
### Crystal structure of ArR-ωTA

<table>
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<th><strong>Data collection</strong></th>
<th>PLP-Lys188</th>
<th>PLP-Lys188</th>
<th>PMP/PLP-D-Ala</th>
<th>PMP</th>
<th>PLP-GABA</th>
<th>PLP-DCS</th>
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<td>In house</td>
<td>In house</td>
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<td>ESRF, ID29</td>
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<tr>
<td>Wavelength (Å)</td>
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<td>0.68</td>
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<td>P4₂/2₂</td>
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<td>Unit cell dimensions, a, b, c (Å)</td>
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<td>80.8, 80.8, 96.2</td>
<td>80.2, 80.2, 95.4</td>
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<td>Resolution (Å)</td>
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<td>185892(25380)</td>
<td>15938 (5896)</td>
<td>29777 (2915)</td>
<td>386564 (38224)</td>
<td>230215 (19418)</td>
<td>41046 (3920)</td>
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<td>20785(2907)</td>
<td>15938 (1500)</td>
<td>15648 (1532)</td>
<td>42508 (4165)</td>
<td>30992 (3063)</td>
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<td>28.9 (3.9)</td>
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<td>98.4 (94.4)</td>
<td>99.8 (99.3)</td>
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<td>Rmerge(%)</td>
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<td>0.11 (0.41)</td>
<td>0.05 (0.39)</td>
<td>0.12 (0.41)</td>
<td>0.05 (0.40)</td>
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### Refinement

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<td>Solvent</td>
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<td>96.8,0</td>
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**Table 2:** Data collection and refinement statistics of the ArR-ωTA structures

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Figure 2: Overall structure of the ArR-ωTA dimer in the orthorhombic crystal form. The structure is shown as a ribbon representation with the two subunits in different colors (green and cyan). The PLP internal aldimines in the two active sites are shown as ball and sticks (carbon, yellow; oxygen, red; nitrogen, blue). One of the "active-site" loops (belonging to the cyan subunit, but complementing the active site of the green subunit) is highlighted in red (in this view, the other, dimer-related loop is located at the back side of the dimer).

Overview of the structure

The overall structure of ArR-ωTA (Figure 2) is highly similar to the structures of the three fungal R-selective ω-TAs characterized up till now (Łyskowski et al., 2014; Sayer et al., 2014; Thomsen et al., 2014). Using the PDBeFOLD server (Krissinel, 2007), the Ca-backbone of a single subunit of ArR-ωTA can be superimposed with those of the fungal (R)-selective ω-TAs with an average rmsd of 1.1 Å for ~297 common residues (average Q-score is 0.74, average sequence identity is 40%). Briefly, the fold of the ArR-ωTA subunit belongs to the transaminase fold type IV (Grishin et al., 1995; Jansonius, 1998) and consists of two α/β domains. Domain I (residues 10-159 and 321-330) contains three α-helices (α1-3) and eight β-strands (β1-7, β16), seven of which form an anti-parallel β-sheet with topology β2-β1-β7-β6-β4-β5-β14. Domain II
(residues 160-320) contains five α-helices (α4-8) and eight β-strands (β8-15). The eight strands form a highly curved central β-sheet of mixed type with topology β8-β9-β10-β15-β14-β11-β12-β13. Significant overall differences between ArR-ωTA and the fungal ω-TAs are mainly restricted to their N-terminal regions. While the fungal ω-TAs contain a long N-terminal a-helix, the first 30 N-terminal residues in ArR-ωTA fold into a long hairpin-like loop.

**Figure 3**: Schematic drawing of the interactions of PLP in the internal aldimine state in the active site of ArR-ωTA. Other residues (not shown) from the other subunit contributing to the active site are Q58*, L61*, H62*, and R138*.

**The internal aldimine state**

The two active sites in the ArR-ωTA dimer are located near the dimer interface. Each active site is formed by residues at the domain interface of one subunit, complemented by residues from domain I of an adjacent subunit (Figure 3 and legend). The conserved catalytic residue Lys188, which is covalently bound to the PLP in the internal aldimine state, is located in the β8-α5 loop of domain II. The pyridine ring of PLP is sandwiched between the backbones of Gly224-Phe225 at its si side, and the side chain of Leu243 at its re side. The Schiff base with Lys188 is formed at the re side of PLP, leaving the si side relatively exposed to the solvent and incoming substrate, a
characteristic feature of the type-IV fold (Sugio et al., 1995; Łyskowski et al., 2014). Other residues interacting with PLP are Tyr67, Trp192 and Asn189 (water-mediated hydrogen bonds with the 3-OH group), Glu221 (salt-bridge with the N1-amino group), Arg248 (salt-bridge with the 5’-phosphate group), Ile246, Thr247, Thr283 (hydrogen bonds with the 5’-phosphate group), and Val69, Asn226 and Arg248 (water-mediated hydrogen bonds with the 5’-phosphate group). The 5’-phosphate group of PLP is bound at the N-terminus of helix α6; the interaction with the helix dipole moment probably contributes to stabilization of the bound co-factor (Sugio et al., 1995; Schneider et al., 2000; Denesyuk et al., 2002). All these interactions with PLP are characteristic of fold-type IV PLP-dependent enzymes, and the amino acid residues concerned are highly conserved in the structurally characterized (R)-selective ω-TAs, as well as in the related fold-type IV aminotransferases d-AAT and BCAT (Peisach et al., 1998a; Goto et al., 2003) (Figure 4).

**Trapping additional states of ArR-ωTA**

Crystal soaks and co-crystallizations were applied to obtain structures of ArR-ωTA in different states (Table 2, Figure 5). Initial soaks of tetragonal crystals with 200 mM D-Ala for 30-60 seconds, immediately followed by flash-cooling, converted crystals from the PLP-internal aldimine to a PMP-bound state, as evident from a complete disappearance of continuous electron density between the cofactor and the side chain of the catalytic Lys288 (Figures 5a and 5b). Similarly, co-crystallization with 100 mM
Figure 4: Structure-based multiple sequence alignment of ArR-ωTA and its close structural homologs.

ωTAat (R-selective ω-TAR from Aspergillus terreus, PDB entry 4CE5), ωTAnh (R-selective ω-TA from Nectria haematococca, PDB entry 4CMD), ωTAaf (R-selective ω-TA from Aspergillus fumigatus, PDB entry 4CHI), DAAT (D-alanine amino transferase from E. coli, PDB entry 4DAA), BCAT (branched chain amino acid transaminase from E. coli, PDB entry 1VE). Colored dots: residues interacting with PLP (green), residues lining the P-pocket (red), residues lining the O-pocket (blue), catalytic lysine (black). The figure was generated using ClustalW (McWilliam et al., 2013) and Consurf (Celniker et al., 2013).

D-Ala resulted in crystals of ArR-ωTA in the PMP-bound state (data not shown). Shorter soaks (10-20 seconds) with D-Ala resulted in a mixed state containing predominantly (∼70%) bound PMP, as well as a small, but significant portion (∼30%) of the PLP-D-Ala external aldimine state (Figure 5c). The presence of continuous electron density extending away from the C4'-amino group of the co-factor clearly indicated the existence of a PLP-D-Ala derivative. Interpretation of the electron density was hindered, though, by the presence of three water molecules in the
active site of PMP-bound ArR-ωTA whose positions are very close to the inferred positions of the Cα, Cβ and carboxyl atoms of PLP-D-Ala (Figures 5b and 5c). The PLP-D-Ala

**Figure 5:** Active site of ArR-ωTA in the tetragonal crystal structures “trapped” at different states. PLP, PMP, co-factor adducts, and the active site residues Lys188 and Tyr67 are shown as sticks, water molecules as spheres; their corresponding electron densities are drawn from 2Fo-Fc composite omit maps (contour level at 1s). (a) Lys188-PLP internal aldimine state. (b) PMP-bound state. (c) Mixed PLP-D-Ala/PMP-bound state (PMP-bound state in grey). (d) PLP-D-cycloserine bound state. (e) PLP-gabaculine bound state. (f) Overlay of the different states using the same coloring scheme as in a-e.
external aldimine structure was constructed by carefully checking the fit of docked PLP derivatives to unbiased omit 2Fo-Fc and Fo-Fc maps, selecting the one with the best fit for subsequent crystallographic refinement. It should be noted, though, that the electron density for the D-Ala adduct of PLP is weak and significantly disordered, probably due to some rotational freedom around the N-Cα bond. Also, it cannot be excluded that the co-factor derivative is present in a mixed external aldimine/ketimine state.

In addition to the PMP-bound and external aldimine states, two inhibitor-bound states were trapped by quick soaks of the tetragonal PLP-bound crystals in D-cycloserine (10 mM, 30 sec, Figure 5d) and gabaculine (5 mM, 10 sec, Figure 5e). All structures were successfully refined at resolutions ranging from 2.3 Å to 1.6 Å (Table 1), and used for further analysis. Similar crystal soaks with L-Ala did not result in a change of the internal aldimine state (data not shown), confirming the strict preference of ArR-ωTA for D-Ala as amino donor.

**PMP-bound and external aldimine states**

Soaking experiments of the catalytically active ArR-ωTA crystals with D-Ala resulted in an ArR-ωTA•PMP/PLP-D-Ala structure that shows several structural changes in the active site. Cleavage of the Lys188-PLP internal aldimine bond and subsequent conversion to the external aldimine and PMP-bound states is associated with a considerable rotation of the PLP pyridine ring of ~30° (Figure 5f), similar to what has been observed in other PLP-dependent enzymes including d-AAT (Sugio et al., 1995), BCAT (Goto et al., 2003) and the fungal (R)-selective ω-TAs (Łyskowski et al., 2014; Sayer et al., 2014; Thomsen et al., 2014). The released Lys188 side chain adopts a conformation, in which its terminal amino group has rotated away from the PLP cofactor, and participates in a water-mediated hydrogen-bonding network with the side chain of Arg248, the main chain of Val69 and the phosphate group of PLP. There is no evidence for a bound pyruvate product in the PMP-bound crystal structure. Instead, at its presumed position, three water molecules are observed, which form a hydrogen-bonding network together with the amino groups of Lys188 and PMP.
The PLP-D-Ala external aldimine structure of ArR-ωTA was analyzed in more detail. The α-methyl group of D-Ala is bound in the P-pocket (a small pocket near the PLP phosphate group (Shin & Kim, 2002; Wybenga et al., 2012)), via van der Waals contacts with the side chains of Val69, Phe122, Thr283 and Ala284 (Figure 6a,b). The α-carboxylate group binds in the O-pocket (near the PLP 3-OH group), adjacent to the side chains of Leu61*, His62*, Tyr67, Trp192 and Phe225. There are strong indications that Arg138' in the adjacent active loop stabilizes the α-carboxylate group by electrostatic interactions (explained below). The D-Ala Ca-H proton points towards the side chain amino group of Lys188, as required for proton abstraction by this residue in the proposed 1,3 prototropic shift, leading to the external aldimine/ketimine conversion and subsequent formation of PMP and pyruvate.

Although the overall conformation of the PLP-D-Ala derivative agrees with the external aldimine state, it should be noted that the D-Ala adduct is not bound in an optimally productive way. The Ca-H bond makes an angle of ~75° with the plane of the PLP ring, instead of 90°, reducing the hyperconjugation effect, which is supposed to facilitate proton abstraction and to steer the reaction towards the ketimine state (Toney, 2014). Moreover, the amino group of the catalytic Lys188 is too far away to allow proton abstraction from the D-Ala Ca atom (3.8 Å). Yet, small rotations around flexible bonds in the PLP-D-Ala external aldimine and Lys188 side chain would be sufficient to produce a productive binding mode, the absence of which perhaps explains the relative stability of the PLP-D-Ala intermediate in the crystal.
Figure 6: External aldimine state of ArR-ωTA. (a) Stereo diagram of the active site with the covalently bound PLP-D-Ala intermediate. (b) Schematic drawing of the interactions of PLP in the active site of ArR-ωTA. The blue and red spheres indicate the O- and P-pockets, respectively.
Chapter 4

Flexibility of active-site loop

Interestingly, weak electron density in the ArR-ωTA●PMP/PLP-D-Ala structure indicates the presence of the active-site loop (residues Gly130-Arg144) from the dimer-related subunit in a closed conformation, with the side chain of Arg138\* pointing towards the carboxylate group of D-Ala allowing formation of a salt bridge (Figure 7a). The closed conformation differs substantially from the conformation of the active-site loop in the orthorhombic structure of ArR-ωTA; the guanidinium head group of Arg138\' has shifted by \( \sim 11 \) Å (Figure 7b). It should be noted that the weak electron density and relatively high atomic B-factors indicate that the active-site loop is still significantly disordered and adopts the closed conformation only in a fraction of the unit cells, probably correlating with the partly occupied PLP-D-Ala state.

Flexibility of the active-site loop, and participation of an arginine residue in substrate binding, has also been discussed for the fungal R-selective ω-TAs (Łyskowski et al., 2014; Sayer et al., 2014; Thomsen et al., 2014). However, the sequence and conformations of the active-site loop in ArR-ωTA differ significantly from those of the fungal ω-TAs (Figures 4 and 7c), and Arg138\* is not conserved in the latter enzymes. Instead, the fungal ω-TAs contain a conserved arginine residue at a different position in the active-site loop (Arg128\' in the ω-TAs from \textit{A. terreus}, Arg126\' in ω-TAs from \textit{N. haematococca} and \textit{A. fumigatus}), which locates near the active site in the fungal structures and perhaps may have a similar role as Arg138\' in ArR-ωTA (Figure 7c). More studies are necessary, however, to establish the exact role of the active-site loop in the R-selective fold type IV ω-transaminases.
Figure 7: Active-site loop in Arr-\(\omega\)TA. (a) Omit 2Fo-Fc electron density of the active-site loop in the Arr-\(\omega\)TA\textbullet PMP/PLP-D-Ala structure. (b) Comparison of the active site loops of the tetragonal (grey) and orthorhombic (cyan) Arr-\(\omega\)TA structures. (c) Comparison of the active site loops of the Arr-\(\omega\)TA structures (cyan, grey) with those of the R-selective \(\omega\)-TAs from \textit{A. terreus} (gold, PDB ID 4CES), \textit{N. haematococca} (green, PDB ID 4CMD) and \textit{A. fumigatus} (violet, PDB ID 4CHI).
Chapter 4

Inhibited states with D-cycloserine and gabaculine

The structures obtained by soaking crystals with the DCS and GABA inhibitors provided further insights into the architecture of the active site pocket of ArR-ωTA and confirmed the catalytic competence of the enzyme in the tetragonal crystals. Both inhibitors clearly formed a covalent adduct with the PLP-cofactor (Figures 5d and 5e), although the electron density for the PLP-GABA derivative indicates some disorder. In both structures the active-site loop is completely disordered, and was therefore not included in the refined models. In the ArR-ωTA•PLP-DCS structure the electron density for the PLP-DCS derivative is in agreement with formation of the final product 3-hydroxyisoxazole (Figure 8). Formation of this aromatic product is the result of

![Diagram showing hydrogen bonding interactions](image)

**Figure 8:** Schematic diagrams showing the hydrogen bonding interactions of the PLP-inhibitor derivatives in the active site of ArR-ωTA (a) PLP-GABA, (b) PLP-DCS. The dashed lines in red show interatomic distances (not H-bonds).

tautomerization of PLP-DCS, after conversion to the ketimine state, which prevents hydrolysis of the PLP-inhibitor derivative (Lepore et al., 2010). The tautomerization reaction requires abstraction of the Cβ-H proton of the cycloserine ring (Lepore et al., 2010). Although the distance between the amino group of Lys188 and the Cβ atom is somewhat large (4.3 Å), the relative orientation of Lys188 is suitable for this residue to act as the catalytic base in the
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Crystal structure of ArR-\(\omega\)TA

tautomerization step. Similarly, distances and orientations of the amino group of Lys188 relative to the \(\text{Ca}\) and \(\text{C'}\) atoms of PLP-DCS are in agreement with a role of this residue as catalytic base/acid during the 1,3-protoprotropic shift leading to formation of the ketimine (Figure 8). The overall conformation of PLP-DCS at the active site of ArR-\(\omega\)TA is similar to that of PLP-DCS in d-AAT from \textit{E. coli} (Peisach \textit{et al.}, 1998b). The hydroxyl group of the 3-hydroxyisoxazole in the PLP-DCS adduct is located in the O-pocket while the cyclic \(\text{C\beta}\) and \(\text{Oy}\) atoms are partly occupying the P-pocket. Nevertheless, there are less interactions with PLP-DCS in ArR-\(\omega\)TA than in d-AAT. In the d-AAT●PLP-DCS crystal structure the 3-hydroxyisoxazole ring of the PLP-DCS derivative forms several direct hydrogen bonds with protein residues in the O-pocket, including Tyr31 (the equivalent of Tyr67 in ArR-\(\omega\)TA ; such interactions are absent in ArR-\(\omega\)TA ●PLP-DCS. The only polar interaction is a water-mediated hydrogen bond between the hydroxyl group of the 3-hydroxyisoxazole ring with \text{His62}\(^*\).

The PLP derivative in the ArR-\(\omega\)TA●PLP-GABA crystal structure was refined as \(m\)-carboxyphenylpyridoxamine phosphate, assuming a similar conversion to a stable final aromatic product as in the ArR-\(\omega\)TA ●PLP-DCS crystal structure. The somewhat poor fit to the electron density indicates that the PLP-GABA derivative is disordered, presumably due to incomplete conversion to the final product. The overall structure is similar to that observed in the \(\omega\)-TA from \textit{N. haematococca} (Sayer \textit{et al.}, 2014), with the \(\gamma\)-carboxyl group located in the O-pocket, while the \(\text{Ce}\) and \(\text{Cz}\) atoms of the phenyl ring make van der Waals contact with residues of the P-pocket. The \(\gamma\)-carboxylate group forms an ion pair with \text{His62}\(^*\) and interacts with a solvent molecule. Intriguingly, the \(\text{C\beta}\) atom in PLP-GABA from which the proton was abstracted during the final reaction step is located in the O-pocket, while the equivalent \(\text{C\beta}\)-atom in PLP-DCS is located in the P-pocket at the opposite side of the \(\text{Ca}\)-atom. With this binding mode of PLP-GABA the hydroxyl group of Tyr67 is closer to the \(\text{C\beta}\) atom than the amino group of Lys188 (3.5 Å versus 4.3 Å), and also more optimally oriented for abstracting the \(\text{C\beta}\)-H proton (Figure 8).
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Mutagenesis of Tyr67 and Arg138

The importance of Tyr67 and Arg138 for the activity of ATA117 was further examined by site-directed mutagenesis (Figure 9). Mutation of Tyr67 to a phenylalanine residue resulted in a nearly inactive enzyme, revealing the crucial importance of this residue for catalysis. The phenyl ring of Tyr67 packs against the carbon atoms of the Lys188 side chain, while the hydroxyl group participates in a hydrogen bonding network to stabilize a water molecule, which in turn forms a hydrogen bond with the 3-OH group of PLP.

Mutation of Tyr67 to a phenylalanine will destabilize the bound water molecule, thereby indirectly affecting the interactions of PLP. Proper hydrogen bonding of the PLP O3 with a water molecule may be important for withdrawing electron density away from the Cα-atom in the PLP external aldimine state, facilitating the Cα-H proton abstraction. Alternatively, residue 67 in the Tyr67Phe mutant may adopt a different conformation from that in the native protein,

![Figure 9: Relative initial rates as calculated from the D-amino acid oxidase coupled assay based on production of H₂O₂ and related to the rate obtained with (R)-methylbenzylamine (1). (2) (R)-4-phenyl-2-butane, (3) (R)-1-(3-chlorophenyl)ethanamine, (4) (R)-1-cyclohexylethylamine, (5) (R)-ethylbenzylamine. No activity was observed using (S)-amines as substrates.](image-url)
thereby changing the direct environment of Lys188. Tyr67 is strictly conserved in the other, fungal (R)-selective ω-TA, as well as in d-AAT (Figure 4), further pointing to an important functional or structural role. Surprisingly, in A. terreus ω-TA the equivalent Y60F mutation resulted in only a ~40-50% reduction of the activity. Thus, further research is needed to establish the role of this residue for the enzyme’s functioning.

Mutation of Arg138 to either an alanine or glutamine residue caused a reduction in activity of ArR-ωTA by about 40-50%. While the reduction in activity agrees with a role of Arg138 in substrate binding and/or catalysis, it is clearly not a crucial role. Probably, exposure to solvent of the α-carboxyl group by moving the active-site loop away from the O-pocket may provide sufficient stabilization of the external aldimine and ketimine states for catalysis to proceed. A similar result has been obtained with the (R)-selective ω-transaminase from A. terreus, which also displayed 40-50% residual activity upon mutation of Arg128 in the active site loop to alanine (Lyskowski et al., 2014).

**Structural insights into substrate specificity and stereo-selectivity**

ArR-ωTA, like other R-selective ω-TAs, is of industrial interest due to its ability to convert a broad range of ketones to their corresponding chiral (R)-amines, using D-alanine as the preferred amino donor. Vice-versa, the enzyme can use chiral (R)-amines as amino donors and convert them to their corresponding ketones, using pyruvate as amino acceptor. Previous characterizations of the substrate scope of ArR-ωTA/ATA117 (Iwasaki et al., 2003; Iwasaki et al., 2006; Mutti et al., 2011; Iwasaki et al., 2012) led to the following observations: (1) the enzyme is 100% stereo-selective: only (R)-amines and D-alanine are reactive as amino donors; (2) the chiral center (the Cα-atom) of the substrate should carry a hydrogen atom and a methyl or ethyl group as its two lowest priority substituents (following the Cahn–Ingold–Prelog rule for defining chirality); (3) the high priority substituent of the Cα-atom should be a carboxylate or an aliphatic moiety, preferably containing a 6-membered aromatic ring. The crystal structures of ArR-ωTA allow an explanation for these observations. The substrate specificity and
enantioselectivity is largely defined by the small hydrophobic P-pocket, which is lined with apolar residues such as Val69, Ala284 and Phe122. Its size is suitable to optimally bind a methyl group. Docking experiments show that an ethyl group still fits in the P-pocket, but that steric strain may force the external aldimine into a non-optimal geometry for proton abstraction by the catalytic lysine, explaining the ~70% reduction in activity towards ($R$)-$\alpha$-ethylbenzylamine compared to ($R$)-$\alpha$-methylbenzylamine as substrate (Figure 9). The position of the catalytic lysine with respect to the P- and O-pockets and PLP defines the stereo-selectivity of the enzyme. Conversion of the PLP internal aldimine by ($S$)-amines or L-alanine would result in an external aldimine intermediate in which the $\alpha$-proton is pointing away from the catalytic lysine, preventing its abstraction and completion of the reaction towards PMP formation. Thus, the small size and apolar nature of the P pocket determine the enzyme’s substrate specificity, while the relative position of the catalytic Lys188 side chain with respect to the P and O pockets, and its location at the re side of the PLP determines the stereo-selectivity of the enzyme.

The O-pocket is considerably larger and more polar than the P-pocket, and, as observed in this study, can bind the carboxylate group of the co-substrate D-Ala with assistance of Arg138' from the adjacent subunit, when the active-site loop adopts a closed conformation. Docking experiments suggest that the O-pocket is suitable for binding the bulky aliphatic moieties of amine substrates when the active-site loop assumes an open conformation (data not shown). Flexibility of the active-site loop and the presence of an arginine residue thus contribute to the dual specificity of the ArR-$\omega$TA O-pocket, bearing some similarity to the arginine-switch in the fold type I ($S$)-selective transaminases (Hirotu et al., 2005).
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Conclusions

The work presented in this chapter has provided a detailed structural and functional understanding of the (R)-selective ω-transaminase from Arthrobacter sp. KNK168. The overall fold of ArR-ωTA is similar to that of other fold type IV enzymes. The crystal structures with various reaction intermediates (of D-Ala, gabaculine, and D-cycloserine) offered detailed insights into the structural basis for the enzyme’s substrate specificity and enantioselectivity. In detail, the P-pocket, which is near the phosphate group of PLP, is lined with the apolar side chains of Val61, Phe122, Thr283, and Ala284. The P-pocket accommodates the smaller substituent of secondary amine substrates. Its small size allows only binding of methyl or ethyl substituents, but not larger ones. The O-pocket, which is near the OH group of PLP, is lined by Leu61*, His62*, Tyr67, Trp192 and Phe225. Our structures show that it can bind the carboxylate group of D-Ala assisted by the side chain of Arg138 from the other subunit. This pocket may also function in binding the bulky substituent of the substrates, similar to what has been observed for (S)-selective ω-transaminases. The position of the catalytic Lys188 side chain, located on the re side of the PLP, determines the enantioselectivity.
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