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An Aminocaprolactam Racemase from *Ochrobactrum anthropi* with Promiscuous Amino Acid Ester Racemase Activity

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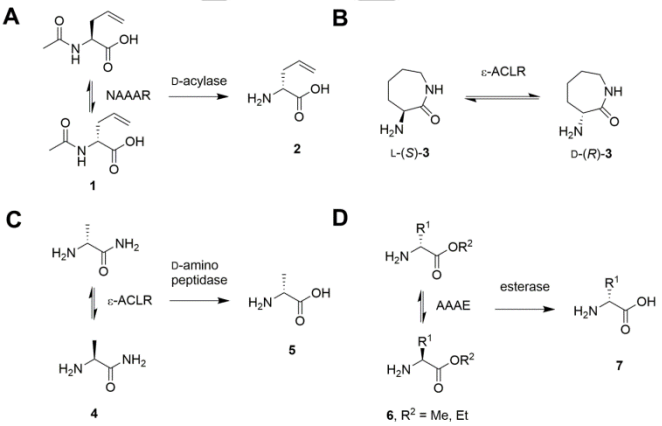
Abstract: The kinetic resolution of amino acid esters (AAEs) is a useful synthetic strategy for the preparation of single enantiomer amino acids. The development of an enzymatic dynamic kinetic resolution (DKR) process for AAEs, which would give a theoretical yield of 100% of the enantiopure product, would require an amino acid ester racemase (AAER), however, no such enzyme has been described. We have identified low AAER activity of 15 U mg^{-1} in a homolog of a PLP-dependent α-amino ε-caprolactam racemase (ACLR) from *Ochrobactrum anthropi.* We have determined the structure of this enzyme, *Oa*ACLR, to a resolution of 1.87 Å and using structure-guided saturation mutagenesis, in combination with a colorimetric screen for AAER activity, we have identified a mutant, L293C, in which the promiscuous AAER activity of this enzyme towards L-phenylalanine methyl ester is improved 3.7 fold.

The preparation of enantiopure amino acids is a process of significant industrial interest. While the preparation of naturally occurring amino acids is often achieved through fermentation,[1-4] other approaches are necessary to provide non-natural products in enantiomerically pure form. Given the excellent selectivity of enzymes, it is no surprise that these have emerged as favoured catalysts for some of these processes. The generation of enantiopure amino acids has been achieved for many years through the kinetic resolution of *N*-acylated amino acids[5,6] amino acid esters (AAEs)[7] or indeed *N*-acylated AAEs,[8] using hydrolases, although the maximum possible yield of these reactions is only 50%. A significant amount of research has therefore been directed towards dynamic kinetic resolution (DKR) processes for the quantitative formation of enantiopure amino acids. In these reactions, the residual enantiomer following resolution is racemised *in situ*, generating further amounts of the enzymatically-favoured substrate enantiomer for hydrolysis. In addition to abiotic methods of racemisation that employ catalysts containing iridium and ruthenium [9,10] enzymes can also be used for this step.[11] In one example, an *N*-acyl amino acid racemase (NAAAR) from *Amycolatopsis* sp. Ts-1–60 has been applied to the dynamic kinetic resolution of *N*-acyl amino acids including *N*-acetyl–D/L-allyl glycine **1** (**Scheme 1A**).[12-15] *In vitro* evolution was used to create a mutant G219D/F323Y of improved activity that could be applied in a 50 g L^{-1} scale resolution of the substrate in combination with an enantiospecific acylase.[15] The activity of this racemase is dependent on two lysine residues acting as proton donor and acceptor and a magnesium ion that coordinates the carboxylate of the amino acid substrate.



Scheme 1. **A**: deracemisation of *N*-acyl allylglycine using *N*-acyl amino acid racemase (NAAAR) and a D-acylase;[15] **B**: Racemisation of α-amino ε-caprolactam by α-amino ε-caprolactam racemase (ε–ACLR);[18] **C**: Deracemisation of L-alanine amide by εACLR and a D-amino peptidase;[20] **D:** proposed deracemisation of amino acid esters using a combination of 'amino acid ester racemase' (AAER) and an enantioselective esterase (this study).

In contrast to NAAAR, many other racemases with activity towards amino acid derivatives are dependent on the cofactor pyridoxal-5'-phosphate (PLP).[16,17] Some of these enzymes have also been applied to DKRs. α-Amino ε-caprolactam racemase (ACLR, **Scheme 1B**[18]) from *Achromobacter obae*, which naturally catalyses the racemisation of the ε-ACL substrate **3**, has been used for the industrial production of L-lysine.[19,20] Asano and co-workers demonstrated that ACLR also displays promiscuous activity towards amino acid amide (AAA) substrates such as alanine amide **4**, and norvaline and phenylalanine amide[21,22] and have applied the enzyme, and a mutant with improved activity,[23] in combination with a D-aminopeptidase from *Ochrobactrum anthropi*, for the dynamic kinetic resolution of AAAs (**Scheme 1C**) to yield D-phenylalanine and its derivatives with yields of >99% and enantiomeric excesses from 16-99% e.e. The structure of ACLR was determined[24] and used in conjunction with previous solution studies[25] to formulate a proposal for the mechanism of the enzyme. We have recently refined the mechanistic hypothesis for ACLR enzymes using high-resolution crystallographic snapshots of the reaction coordinate with the ACLR from *Rhizobium frerei* (*Rf*ACLR).[26] These studies hint at significant ligand dynamics during the racemisation of ε-ACL, and suggest that Asp210 and Lys267 act as the proton donor/acceptor in the racemisation mechanism.

In an extension to the DKR technology, Wegman and co-workers[27] demonstrated that, by taking advantage of the faster rate of amino acid ester (AAE) racemisation compared to the amide, enantiopure amides can be prepared directly from racemic acid esters by lipase catalysed ammoniolysis in the presence of aldehydes such as pyridoxal. However, aldehydes such as pyridoxal are expensive and not readily recycled. Therefore, given the commercial importance of amide bond formation from unnatural amino acids and their

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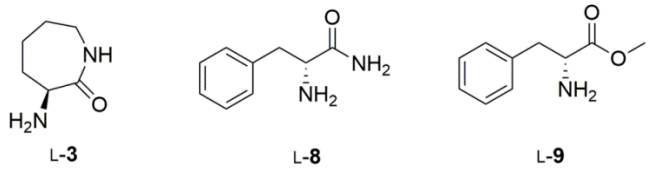
derivatives, we envisaged that the development of 'amino acid ester racemases' (AAERs) could be highly valuable, and that such an enzyme may be discovered through an examination of the catalytic promiscuity of ACLRs. Catalytic promiscuity of enzymes is a topic of significant contemporary interest,[28,29] as the identification of even low activity can lead to improved catalysts through protein engineering techniques such as directed evolution. In this report we demonstrate low native activity of an ACLR homolog from *Ochrobactrum anthropi* (*Oa*ACLR) for AAE racemisation, and the structure guided engineering of the enzyme, which has given a variant, L293C, displaying higher activity for the racemisation of L-phenylalanine methyl ester.

Target Selection

In an effort to identify an enzyme competent for the racemisation of amino acid esters, we cloned and expressed three ACLR homologs (*Ao*ACLR, *Rf*ACLR and *Oa*ACLR). *Rf*ACLR and *Oa*ACLR (Uniprot code Q06K28) were identified as putative ACLRs by sequence homology search using *Ao*ACLR as a model (Details of cloning, expression and purification can be found in the **Supporting Information**). We have examined the ACLR activity of *Rf*ACLR extensively in a previous study, and used it as a structural target to examine ACLR mechanism in general.[26] *Oa*ACLR shares 53 and 52% sequence identity with *Ao*ACLR[18] and *Rf*ACLR[26] respectively (**Figure S1**), and was annotated as an 'amino acid amide racemase with transaminase activity'. *Oa*ACLR had previously been identified as an ACLR by Boesten and co-workers in a patent by DSM[30] and also by Asano and co-workers as part of a wider study of ACLR homologs in 2015,[31] although was not purified in the latter case. An additional ACLR from *Ochrobactrum anthropi*, Oant_4493, had also been identified as having ACLR activity towards D-ACL of 1225 U mg^{-1} by Asano and co-workers,[31] although *Oa*ACLR is a different enzyme, containing, among other differences, an alanine residue in place of a tryptophan in position 49 (*Ao*ACLR numbering) of the sequence (**Figure S1**). Otherwise all three enzymes share significant homology throughout the length of the polypeptide chain. We tested *Ao*ACLR, *Rf*ACLR and *Oa*ACLR for racemisation activity towards α–amino-ε-caprolactam **3**, phenylalanine amide **8** and phenylalanine methyl ester **9** (**Scheme 2**) using an HPLC assay (Details of HPLC assay can be found in the **Supporting Information**).



L-**3** L-**8** L-**9**

Scheme 2. Substrates used in this study: L-**3** – ε-ACL; L-**8** – L-Phenylalanine amide; L-**9** = L-Phenylalanine methyl ester.

*Ao*ACLR and *Rf*ACLR showed similar activity towards ε-ACL and phenylalanine amide. *Oa*ACLR displayed approximately ten and four-fold reduced specific activity compared to *Rf*ACLR for the racemisation of ε-ACL and phenylalanine amide respectively. However, *Oa*ACLR also displayed activity towards the ester substrate **3** (15.3 U mg^{-1}; **Table 1**), whereas *Ao*ACLR and *Rf*ACLR did not.

Table 1. Comparison of specific activity of *Oa*ACLR and *Rf*ACLR towards L-ε-ACL **3**, L-phenylalanine amide **8** and L-phenylalanine methyl ester **9**.

	Specific activity toward Substrate (U mg^{-1})		
Enzyme	L-**3**	L-**8**	L-**9**
*Ao*ACLR	30117 ± 972	11.9 ± 5.2	0
*Rf*ACLR	26469 ± 5425	20.4 ± 0.8	0
*Oa*ACLR	2337 ± 291	5.2 ± 1.6	15.3 ± 0.7

This difference prompted us to examine differences between these ACLRs using X-ray crystallography, in order to identify residues that might explain the difference in reactivity and suggest target residues for engineering improved amino acid ester racemase activity in *Oa*ACLR.

Structure of OaACLR

We crystallised *Oa*ACLR and determined the structure of the enzyme in complex with PLP to a resolution of 1.87 Å (Details of Crystallisation and **Table S1** featuring Data Collection and Refinement statistics can be found in the **Supporting Information**). The structure featured four molecules in the asymmetric unit, constituting two dimers, with two active sites at the dimer interface containing PLP (**Figure 1A**). There was clear continuous density extending from the side chain of Lys267 to the PLP molecule in all active sites, indicative of formation of the internal aldimine complex. Although co-crystallisation was carried out with different ligands (L-ACL **3**, L-$PheNH_2$ 8, L-PheOMe **9**, and L-Phenylalanine) under numerous conditions, additional ligand density proved insufficient for modelling and refinement for any of these compounds. Due to the high sequence homology between ACLRs (**Figure S1**), the overall structure of the *Oa*ACLR monomer, as well as the structure of the active site, is highly conserved with respect to the published monomer structures of *Ao*ACLR (2ZUK[24]) and *Rf*ACLR (*e.g.* 5M49 [26]), with rmsds of 0.89 Å and 0.84 Å over 415 C-α atoms respectively.

Conserved residues in addition to Lys267 included Lys241, implicated in substrate carbonyl recognition in *Rf*ACLR, and Asp210, which, with Lys267 are thought to be the proton donors/acceptors in the racemase mechanism. Trp436 at the C-terminus of each sequence, which appears to form a gate over the active site in *Rf*ACLR, is conserved but does not feature in the electron density for the *Oa*ACLR structure. Indeed density for this residue in the *Rf*ACLR structures only featured when ligand was present.[26] Tyr139, which stacks against the plane of the ε-ACL substrate is conserved, but Trp49, which in *Ao*ACLR and *Rf*ACLR stacks against the other face of the substrate, is replaced by Ala51 in *Oa*ACLR, creating more room in the active site. Additionally, (B)Met293 from the neighbouring monomer in both *Ao*ACLR and *Rf*ACLR enzymes, which is situated beneath the ε-ACL ligand at the base of the binding pocket, is replaced by Leu293 in *Oa*ACLR. These mutations are also to be found, although not together, in other ACLR homologs recently expressed by Asano and co-workers.[31] The differences in *Oa*ACLR, coupled with its distinctive AAER activity, immediately suggested sites for mutation that would both investigate the distinctive ability of *Oa*ACLR to catalyse the amino acid ester racemisation reaction, but also for saturation mutagenesis that may improve the promiscuous activity.

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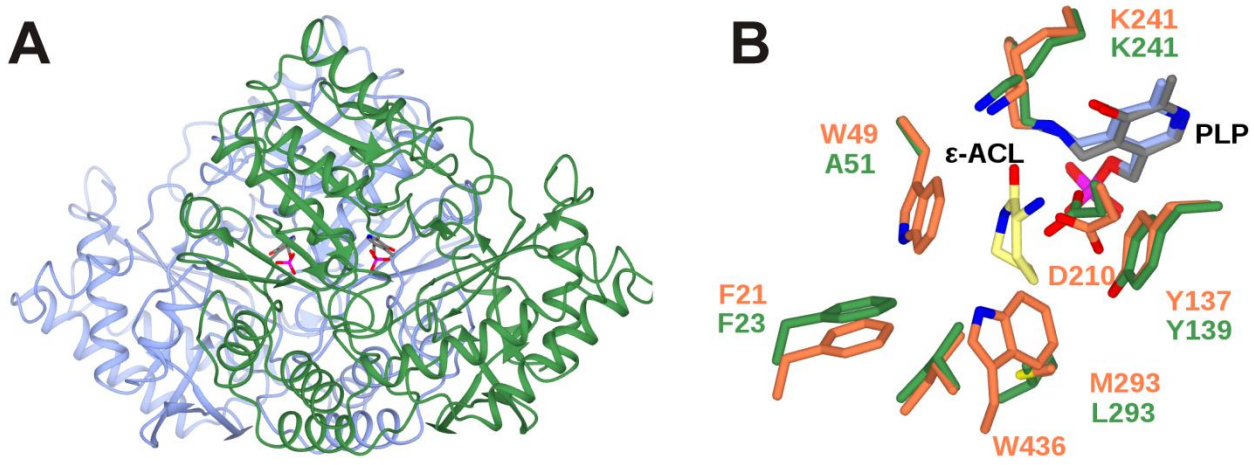

Figure 1. **A**: Structure of *Oa*ACLR dimer with subunits shown in ribbon format in light blue and green. PLP is shown in cylinder format, with carbon atoms in grey, in two active sites at the dimer interface; **B**: Superimposition of active site residue side-chains of *Rf*ACLR internal aldimine complex with ε-ACL (PDB code 5M49,[26]) and *Oa*ACLR-PLP complex with carbon atoms in coral and green respectively. PLP atoms are shown in grey and the ε-ACL carbon atoms from 5M49 in yellow.

A screen for AAAE activity

In order to embark on *in vitro* evolution studies of even conservatively-sized libraries, it was necessary to develop a plate-based colorimetric screen for AAER activity. This was based upon the assay developed for NAAAR evolution devised by Campopiano and co-workers.[15] In this assay, D-phenylalanine methyl ester (D-PheOMe D-**9**) was used as the substrate and racemised by variants of *Oa*ACLR. In the next step, only L-PheOMe L-**9**, which is the result of AAAE activity, was hydrolysed by an L-alcalase from *Bacillus licheniformis*[32] to L-phenylalanine (L-Phe). The concentration of L-Phe could then be determined spectrophotometrically through the action of an L-amino acid oxidase (L-AAO), coupled to the horseradish peroxidase catalysed transformation of *o*-dianisidine, dependent upon the hydrogen peroxide released in the L-AAO reaction (**Scheme S1**).

Identification and characterisation of the L293C mutant

Saturation mutagenesis libraries at positions 51 and 293 were generated using inverse PCR-based mutagenesis methods using NNK degenerate primers. Two libraries, each of 96 mutants, were screened using the assay described. Upon comparing the activity of the wild-type and mutants from the library only one mutant in position 293 could be identified with a significant increase in specific activity for racemisation of L-**9**. None of the tested mutants of the library in position 51 gave improved activity over the wild-type. The improved mutant was identified as *Oa*ACLR-L293C and purified (as described in the **Supporting Information**). Interestingly, the purification yield of *Oa*ACLR-L293C was improved 1.7-fold over that of the wild-type enzyme (5 mg L^{-1} compared to 3 mg L^{-1}). The activity of the purified *Oa*ACLR-L293C was determined for L-**9** using the HPLC assay described. Kinetic parameters were determined by measuring the racemisation activity at concentrations up to 60 mM of L-**9**. The K_m values recorded for the wild-type and *Oa*ACLR-L293C were 2.8 mM and 5.6 mM respectively and the V_{max} values were 0.05 μM s^{-1} and 0.34 μM s^{-1} (**Figure 2**). The catalytic efficiency (k_{cat}/K_m) was increased 3.7 fold for the *Oa*ACLR-L293C mutant compared to the wild-type enzyme, from 0.007 μM^{-1} s^{-1} to 0.026 μM^{-1} s^{-1}. In addition, the *Oa*ACLR-L293C mutant was less susceptible to inhibition, with only 15% loss of activity observed at the highest tested substrate concentration, *versus* 63 % loss of activity for wild-type *Oa*ACLR. Although the reason for the improved activity of the Leucine-Cysteine mutant is not clear, it is not attributable to the introduction of an alternate acid-base into the active site, as a L293C/D210A mutant was shown to be inactive (data not shown).

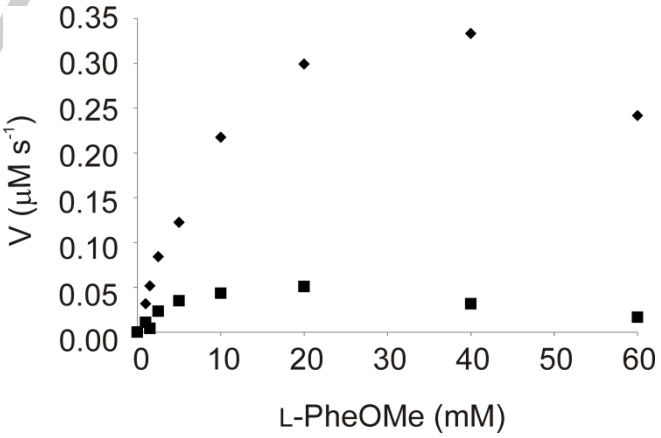

Figure 2. Specific activity of *Oa*ACLR WT (■) and L293C mutant (♦) measured at increasing concentrations of L-PheOMe (L-**9**).

Conclusion

The structural diversity of desired amino acid products in industry dictates that complementary processes must be sought for the generation of these compounds in single enantiomer form. The description of an amino acid esterase activity in this report opens up

new possibilities for the identification of similar activities in homologs and mutants for other amino acid substrates, with the goals of incorporation into preparative dynamic kinetic resolution processes for the production of pharmaceutical intermediates.

Supporting Information

Amino acid sequences, PCR primers, gene cloning, mutation, expression and protein purification protocols; biotransformations, HPLC analysis and plate screen development; Protein crystallisation, data collection and refinement.

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Keywords: Biocatalysis; Racemases; Pyridoxal phosphate; Amino acids; Amino acid amides; Amino acid esters.

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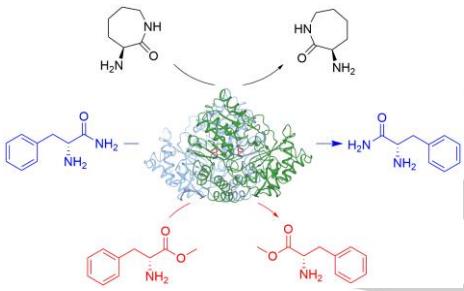
Layout 1:

COMMUNICATION

α-Amino ε-Caprolactam Racemase (ACLR) from Ochrobactrum anthropi *catalyzes the racemization of ACL, but also phenylalanine amide and phenylalanine methyl ester, the latter an unprecedented substrate for ACLRs. We have determined the structure of OaACLR and used this to engineer a mutant with a 3.7-fold greater activity than the wild-type for the amino acid ester racemization.*



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