Snapshots of the Catalytic Cycle of the Industrial Enzyme α-Amino-ε-Caprolactam Racemase (ACLR) Observed Using X-ray Crystallography

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ABSTRACT: α-Amino-ε-Caprolactam Racemase (ACLR) is a PLP-dependent enzyme exploited in industry for the racemization of amino acid amides in dynamic kinetic resolutions that produce homochiral amino acids. We report high-resolution structures of wild-type and variant ACLRs from *Rhizobium freirei*, generated using the chiral substrate ACL and representative of covalent intermediates in the catalytic cycle. These complexes substantiate suggestions of a two-base mechanism, in which the PLP-binding K267 and D210 serve as proton donors in the racemization, and constitute a robust basis on which to engineer ACLRs for improved activity as industrial biocatalysts.

KEYWORDS: PLP, racemase, amino acids, enzyme mechanism, biocatalysis

The use of enantiopure amino acids in the fine chemical industry requires the development of efficient synthetic routes for their production. Proteinogenic amino acids are easily synthesized by many processes, including microbial fermentation,1 but other amino acids, including D-isoforms, can also be produced using biotransformations.2 One elegant method for the production of such enantiopure amino acids is through the enzyme-mediated dynamic kinetic resolution (DKR) of amino acids or their derivatives.3-5 In one possible route, the enantioselective enzymatic hydrolysis of racemic amino acid amides (AAAs) by an amidohydrolase can lead to single enantiomer amino acid products. The inclusion of an AAA racemase enzyme in the reaction mixture serves to increase the yields to a theoretical maximum of 100%, through generation of the favoured enantiomer from the residual, disfavoured one. A potent racemase biocatalyst for application in the DKR of AAAs is the PLP-dependent α-amino-ε-caprolactam racemase from *Achromobacter obae* (*Ao*ACLR;6-8). *Ao*ACLR has been used for the industrial production of L-lysine from the relevant cyclic amino acid amide, α-amino-ε-caprolactam (ACL) **1** (**Scheme 1**).9 Subsequently, other acyclic AAAs were reported to be substrates for *Ao*ACLR, including L-threonine, L-norleucine and L-phenylalanine amide,10 each of which has been resolved using *Ao*ACLR in combination with aminopeptidase enzymes.11,12



Scheme 1. Racemisation of α-amino-ε-caprolactam by ACLR.

The structure of *Ao*ACLR, determined by Yamane and co-workers,13 revealed a dimeric Type I PLP fold enzyme, in which the active site was composed of residues from two protomers at the dimer interface. A further structure of *Ao*ACLR in complex with the inhibitor ε-caprolactam permitted the formulation of a hypothesis for a ‘two-base’ mechanism of racemization in which residues Lys267 and Tyr137 were proposed as residues for the protonation of a planar quinonoid intermediate at the *re-* and *si-* faces of the intermediate respectively, giving rise to the L-(*S*)- or D-(*R*)- enantiomers of the amide product. The two-base hypothesis contradicted the conclusion of previous deuterium labelling experiments, in which a single-base, presumably Lys267, was suggested to be able to protonate the intermediate from either face of the quinonoid.14



Figure 1. A: Internal aldimine complex of *Rf*ACLR, complexed with L-ACL (D-ACL could also be modelled with equivalent occupancy; the deposited structure features a mixture of both with equal occupancy. A single enantiomer is presented here for simplicity). B: Geminal diamine complex of D210A variant of *Rf*ACLR, in which the chiral carbon has the (*R*)- configuration C: External aldimine complex of K241A variant of *Rf*ACLR, in which the chiral carbon again has the (*R*)- configuration. C-alpha and side-chain carbon atoms are shown in green; PLP and ACL carbon atoms in grey and yellow respectively. The side chain of W49, which forms the opposite wall of the aromatic cage with Y137, has been omitted for clarity. Electron density in each frame corresponds to the *F*o-*F*c omit map at a level of 3 obtained prior to refinement of ACL, the atoms of which have been added for the Figure. Interactions between ACL and side chains of *Rf*ACLR are indicated by dashed lines with distances shown in Ångstroms. Stereo images for these and alternate views are given in the Supporting Information (**Figure S5A-F**).

However, the occupancy of the ligand in the structure,13 and the resolution of the complex (2.4 Å), coupled with the absence of a structure in complex with the chiral substrate, militate against a definitive assignment of the roles of active site residues in the racemization mechanism. In this report, we present a study of the ACLR from *Rhizobium freirei* (*Rf*ACLR), with high resolution structures of the enzyme and variants illustrating internal and external aldimine and *gem*-diamine intermediates with the natural substrate ACL, which serve to clarify the identity and probable role of conserved active site residues in these racemases.

*Rf*ACLR was identified by genome mining from amongst a number of possible ACLRs. The enzyme has 84% sequence identity with *Ao*ACLR (**Figure S1**). A synthetic gene, with codons optimized for expression in *E. coli*, was cloned and over-expressed and the enzyme purified using nickel affinity and size exclusion chromatography (Details are in the Supporting Information). A solution of purified *Rf*ACLR was assayed against L-ACL using an HPLC-based method, and catalyzed the complete racemization of 50 mM substrate in 15 min (**Figure S2**).

The *Rf*ACLR structure with PLP (*Rf*ACLR-PLP), determined to a resolution of 1.62 Å, revealed a large amount of conservation of the active site with respect to the *A. obae* enzyme as expected from the high sequence identity. The first ligand complex of *Rf*ACLR (*Rf*ACLR-PLP-ACL), obtained by co-crystallization with L-ACL, and refined to 1.51 Å, features the internal aldimine structure in which K267 forms the common Schiff base link with the aldehyde of PLP. The superior resolution of the data revealed the presence of two forms of ACL, which were bound coplanar with the side chains of Y137 and W49 forming an ‘aromatic cage’ that leads to the PLP. The density was modelled and refined as a mixture of enantiomers with equal occupancy, although the average *B*-factor for the D-, of 20, was higher than that for the L-, of 15. When compared to the *Ao*ACLR-ε-caprolactam inhibitor complex with *Ao*ACLR deposited by Yamane (2ZUK; *13*), a major difference in the conformation of the ligand, and a reorientation of the side-chain of D210 were observed, each of which establish alternate roles for active site residues in ligand recognition to those previously suggested. While the carbonyl groups of ε-caprolactam and L-ACL in 2ZUK and *Rf*ACLR-PLP-ACL are superimposable, each making a hydrogen bond with the ε-amino group of K241, the ligand in *Rf*ACLR-PLP-ACL is rotated approximately 180° such that the endocyclic nitrogen makes an H-bond with the side chain hydroxyl of T295 in the ‘B’ subunit (**Figure 1A;** a superimposition of *Rf*ACLR-PLP-ACL and 2ZUK is shown in **Figure S3**). Interestingly, in their publication, Yamane and co-workers also recognized that their ligand could indeed be modelled with a similar orientation (*13*). D210, observed in 2ZUK to point away from the active site, now interacts at a distance of 2.7 Å with the exocyclic amine of L-ACL; previously this residue had been thought to orient the endocyclic nitrogen of the amide substrate.13 The orientation of the amide bond is supported by its persistent residual density in the omit map, even at a level of 5.

This detailed view of ligand binding in the *Rf*ACLR-PLP-ACL complex prompted us to make mutations in an effort to disrupt racemization activity. The following description of the variant structures makes reference to intermediates shown in a postulated mechanism for *Rf*ACLR activity shown in **Scheme 2**.Mutation of Y137 to phenylalanine resulted in a Y137F variant that was not expressed in the soluble fraction of the *E. coli* strain employed. Mutation of D210 to alanine resulted in variant D210A that was poorly active for the racemization of both L- and D-ACL (<0.001% and 0.04% relative to wild-type respectively).



Scheme 2. A proposed mechanism for *Rf*ACLR activity, in which the configuration of the L-enantiomer of ACL is inverted to the D-, based on X-ray crystallography data. Attack of the L-ACL at the internal aldimine I results in a geminal diamine II with L- or (*S*)-configuration; the bond between K267 and PLP is broken and the (*S*)-form of the external aldimine III results. III is deprotonated at the chiral carbon by K267 to yield the quinonoid intermediate IV, which is protonated from the opposite face of the ligand to give the D- or (*R*)- form of the external aldimine V. Attack of K267, having been most likely deprotonated by K241 (not shown), at the imine bond of V releases the D-enantiomer of ACL *via* a geminal diamine intermediate VI of (*R*)- configuration.

Incubation of the D210A variant with L-ACL turned the ordinarily yellow enzyme solution pink. Analysis of the complex by UV spectrophotometry (**Figure S5**) confirmed partial formation of a quinonoid intermediate **IV** (Scheme 2), as indicated by the increase in a peak at 510 nm.15 The quinonoid intermediate is thought to be stabilized by interaction of the PLP pyridine nitrogen with D238, conserved between *Ao*ACLR and *Rf*ACLR.13 The structure of the D210A variant that had been co-crystallized with L-ACL (*Rf*ACLR-D210-ACL), and refined to 1.50 Å resolution, revealed ligand density again consistent with the presence of ACL. However, in this complex, the ACL was rotated approximately 90° from the conformation in the internal aldimine complex *Rf*ACLR-PLP-ACL, indicative of the change in ligand conformation required for the interaction between the exocyclic amine of ACL and PLP. Additional electron density in the omit map suggested the presence of a covalent intermediate. However, this density was not representative of the quinonoid intermediate; rather it suggested attachment of PLP both to K267 and also to the exocyclic amine of the ligand (**Figure 1B**). This was modelled as the geminal diamine **VI** (discussed below) that is intermediate in the reaction coordinate between the attack/release of the substrate/product at the internal aldimine **I** and the formation of the external aldimine intermediate **V**. However, despite the low activity of the variant for racemization of L- ACL, the stereochemistry at the chiral carbon of ACL in the structure is clearly (*R*)-. This is presumed to arise from complexation of the enzyme with (*R*)-D-ACL that has accumulated as a result of the residual activity of the variant or background racemization (measured to be approximately 2.5% over 24 h) in the crystallization medium during the time taken for crystal growth (up to 5 d). The ligand conformation in the *Rf*ACLR-D210-ACL complex places the -amino group of K267 3.7 Å from the chiral carbon and the -amino group of K241 2.8 Å from the carbonyl oxygen of ACL respectively. The phenolic hydroxyl of Y137 was 4.3 Å distant from the chiral carbon of ACL, but adjacent to the lower methylene groups in the ACL ring, and seems poorly placed to serve as a proton donor in the racemization mechanism as previously suggested.13

Mutation of K241 to alanine gave a variant K241A which, while compromised in its ability to catalyze racemization, was still active (2.5% relative to wild-type). Co-crystallization of the K241A variant with L-ACL furnished another covalent complex structure (*Rf*ACLR-K241A-ACL, **Figure 1C**), in this case featuring an external aldimine intermediate. In this complex the ACL ligand was now rotated further than seen in the D210A variant, to be orthogonal to the plane of the aromatic cage. The chiral carbon of the external aldimine was again clearly of the (*R*)- configuration, and was situated between the side chains of D210 and K267 at distances of 2.8 and 4.2 Å from the carboxylate and -amino groups of those residues respectively, with the former ideally positioned for proton donation to the *si-*face of the presumed planar intermediate. The D210 side chain has moved relative to its position in the *Rf*ACLR-PLP-ACL internal aldimine complex, to be closer to the ligand, as a result of a movement in loop 208-214 that also results in a new stabilizing H-bond interaction between the side chains of S209 and E396. This complex substantiates the suggestion16 that D210 and K267 are the ‘two bases’ involved in the racemization mechanism.

A summary of a mechanism of action suggested by these results is shown in Scheme 2. The *Rf*ACLR-PLP-ACL illustrates the active site prior to attack of the exocyclic amine of ACL on internal aldimine **I**. It appears that, given the overall high activity of wild-type *Rf*ACLR, no reaction intermediates have been isolated, and co-crystallization with L-ACL has resulted in ligand density representative of a mixture of enantiomeric substrate/products in the active racemase. However, given the orientation of the ACL in this complex, it is clear that the ligand would have to rotate in order to bring the exocylic amine of ACL sufficiently close to the internal aldimine bond for the displacement reaction. There is substantial evidence of this rotation in the other structures, including the *Rf*ACLR-D210-ACL complex, in which the exocyclic amine of ACL is now clearly observed making the imine link to PLP. However, additional continuous density from K267 to PLP, plus the (*R*)- configuration at the chiral carbon, suggests that this complex represents the geminal diamine **VI** in Scheme 2. The *Rf*ACLR-K241A-ACL complex, with no additional density connecting K267 and PLP, is more representative of the external aldimine **V**, in which K267 is poised to displace the (*R*)- ligand from the PLP. The arrangement of chiral centre and protic residues in *Rf*ACLR-K241A-ACL is now reminiscent of that observed for both alanine racemase, in which relevant lysine and tyrosine side-chains are 3.5 and 4 Å from the chiral carbon of the substrate,17 and *N*-acyl amino acid racemase,18 in which two lysine residues on opposite sides of the chiral centre serve as acid-base in the racemization of derivatized amino acids including *N*-acyl methionine.

Taken together, the structures of *Rf*ACLR presented herein provide further evidence of the function of active site residues in substrate recognition in ACLRs and are strongly suggestive of D210 and K267 as protic residues responsible for a ‘two-base’ racemization mechanism in ACLRs. This provides further support for the more recent hypothesis proposed by Asano and co-workers (*16*). This is in contrast to the single-base mechanism suggested on the basis of deuterium kinetic isotope effects performed by Ahmed and co-workers previously (*14*), but the structures suggest that no single amino acid is well positioned to fulfil the dual role of proton delivery at either face of the substrate(s). The detailed description of ligand binding and reaction intermediates will assist the rational redesign of ACLRs for improved activity against non-native substrates, with a view to further improved applications in preparative biocatalysis.

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Author Contributions

The manuscript was written through contributions of all authors and all authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

Supporting Information

Cloning and expression of *Rf*ACLR gene and mutants; Purification and crystallization protocols; Tables on X-ray data collection and refinement; HPLC and UV assay details. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

PLP, Pyridoxal-5’-phosphate; ACLR, α-Amino-ε-Caprolactam Racemase; ACL, α-Amino-ε-Caprolactam; AAA, amino acid amide.

REFERENCES

(1) Hermann, T. *J. Biotechnol.,* **2003**, 104, 155-172.

(2) Yagasaki, M.; Ozaki, A. *J. Mol. Catal. B-Enzym*., **1998**, 4, 1-11.

(3) Soriano-Maldonado, P.; Las Heras-Vazquez, F. J.; Clemente-Jimenez, J. M.; Rodriguez-Vico, F.; Martinez-Rodriguez, S. *Appl. Microbiol. Biotechnol.*, **2015**, 99, 283-291.

(4) Brown, S. A.; Parker, M-C.; Turner, N. J. *Tetrahedron: Asymm*. **2000**, 11, 1687-1690.

(5) Baxter, S.; Royer, S.; Grogan, G.; Brown, F.; Holt-Tiffin, K. E.; Taylor, I. N.; Fotheringham, I. G.; Campopiano, D. J. *J. Am. Chem. Soc.*, **2013**, 134, 19310-19313.

(6) Ahmed, S. A.; Esaki, N.; Soda, K. *FEBS Lett.*, **1982**, 150, 370-374.

(7) Ahmed, S. A.; Esaki, N.; Tanaka, H.; Soda, K. *Agric. Biol. Chem.*, **1983**, 47, 1887-1893.

(8) Ahmed, S. A.; Esaki, N.; Tanaka, H.; Soda, K. *Agric. Biol. Chem.*, **1985**, 49, 2991-2997.

(9) Fukumura, T. *Agric. Biol. Chem.*, **1977**, 8, 1327-1330.

(10) Asano, Y.; Yamaguchi, S. *J. Mol. Catal. B-Enzym*., **2005**, 36, 22-29.

(11) Asano, Y.; Yamaguchi, S. *J. Am Chem. Soc.*., **2005**, 127, 7696-7697.

(12) Yamaguchi, S.; Komeda, H.; Asano, Y. *Appl. Environ. Microbiol.*, **2007**, 73, 5370-5373.

(13) Okazaki, S.; Suzuki, A.; Mizushima, T.; Kawano, T.; Komeda, H.; Asano, Y.; Yamane, T. *Biochemistry*, **2009**, 48, 941-950.

(14) Ahmed, S. A.; Esaki, N.; Soda, K. *Biochemistry.*, **1986**, 25, 385-388.

(15) Sun, S.; Toney, M. D. *Biochemistry*, **1999**, 38, 4058-4065.

(16) Payoungkiattikun, W.; Okazaki, S.; Nakano, S.; Ina, A.; H-Kittikun, A.; Asano, Y. *Appl. Biochem. Biotechnol*., **2015**, 176, 1303-1314.

(17) Watanabe, A.; Yoshimura, T.; Mikami, B,; Hayashi, H.; Kagamiyama, H.; Esaki, N. *J. Biol. Chem.*, **2002**, 277, 19166-19172.

(18) Thoden, J. B.; Taylor-Ringia, E. A.; Garrett, J. B.; Gerlt, J. A.; Holden, H. M.; Rayment I. *Biochemistry*, **2004**, 5716-5727.

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