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An Improved Racemase/Acylase Biotransformation for the Preparation of Enantiomerically Pure Amino Acids**

Scott Baxter,^{1,5} Sylvain Royer,² Gideon Grogan,³ Fraser Brown⁵, Karen E. Holt-Tiffin,⁴ Ian N. Taylor,⁴ Ian G. Fotheringham⁵ and Dominic J. Campopiano^{1,*}

^[1]EaStCHEM, School of Chemistry, Joseph Black Building, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JJ, UK.

^[2]Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY, U.K.

^[3]York Structural Biology Laboratory, Dept of Chemistry, University of York, York, YO10 5DD, U.K.

^[4]Chirotech Technology Centre, Dr Reddy's Laboratories Ltd, 410 Cambridge Science Park, Cambridge, CB4 0PE, U.K.

^[5]Ingenza Ltd, Wallace Building, Roslin Biocentre, Roslin, EH25 9PP, U.K.

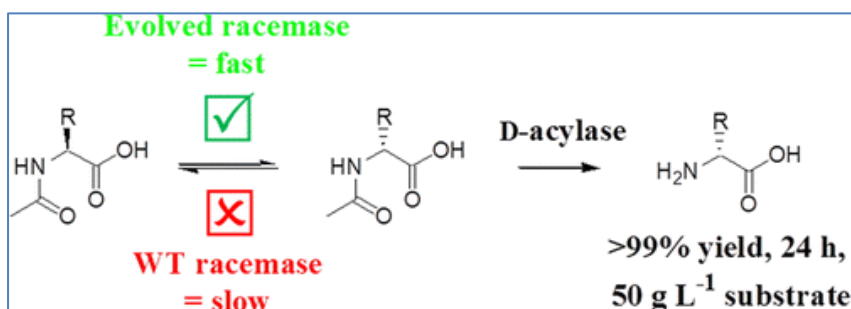
[*]Corresponding author; e-mail: Dominic.Campopiano@ed.ac.uk

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Supporting information:

Descriptions of experimental procedures. This material is available free of charge via the internet at <http://pubs.acs.org>

Graphical abstract:



Abstract

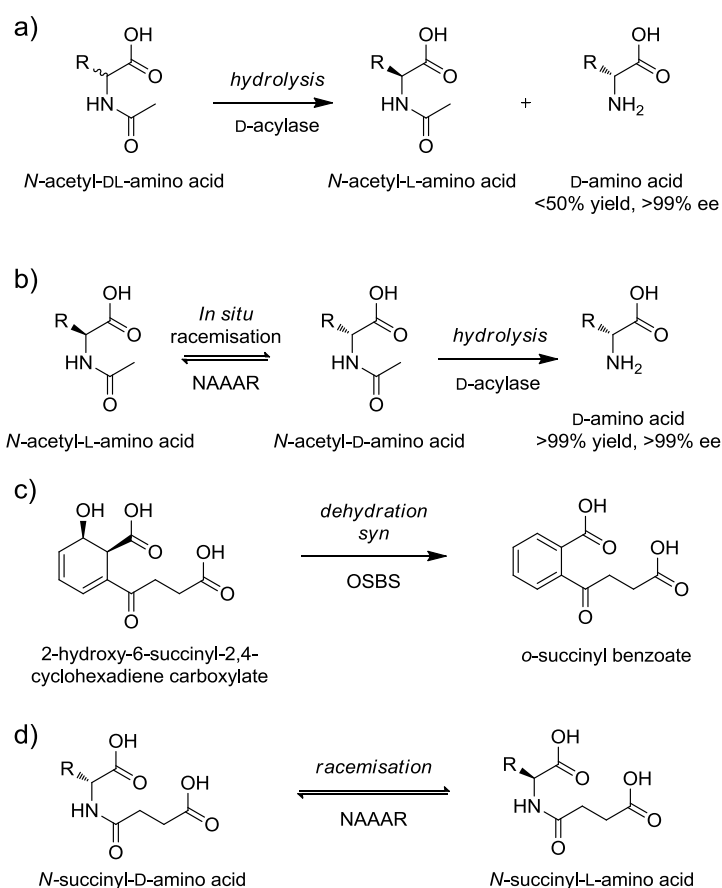
Using directed evolution, a variant *N*-acetyl amino acid racemase (NAAAR G291D/F323Y) has been developed with up to five fold higher activity than the wild-type on a range of *N*-acetylated amino acids. The variant has been coupled with an enantiospecific acylase to give a gram scale dynamic kinetic resolution which allows 89% conversion of *N*-acetyl-DL-allylglycine into D-allylglycine in 18 hours at suitably high substrate concentrations for industrial use (50 g L⁻¹). This is the first example of NAAAR operating under conditions which would allow it to be successfully used on an industrial scale for the production of enantio-pure α -amino acids. X-ray crystal analysis of the improved NAAAR variant allowed a comparison with the wild-type enzyme. We postulate that a network of novel interactions that result from the introduction of the two side-chains is the source of improved catalytic performance.

Introduction

Kinetic resolutions (KRs) that employ stereo-selective acylases are commonly used for the production of both L- and D- α -amino acids from their *N*-acetylated-DL- starting materials.¹⁻⁴ However, a drawback in this process is the need for repeated chemo-catalytic racemisation steps of the non-desired enantiomer to achieve yields >50% (Scheme 1a). These racemisation steps, if performed at all, are typically implemented under harsh chemical conditions resulting in extra cost and waste. A solution to this problem would be a dynamic kinetic resolution (DKR), driven by an *in situ* racemisation step that would allow yields to approach 100% (Scheme 1b). A cheap and “green” catalyst for this racemisation would be an enzyme, such as *N*-acyl amino acid racemase (NAAAR) from the actinobacterium *Amycolatopsis* sp. Ts-1-60. This enzyme has many desirable features in that it requires no organic cofactor, is active at high temperatures, accepts a wide range of amino acid substrates and suffers no substrate or product inhibition up to 300 mM. Unfortunately, the activity of NAAAR with the desired *N*-acetyl substrates is too low for commercial use. To amend this, we have used directed evolution to generate NAAAR variants with increased racemase activity towards *N*-acetyl amino acids. Directed evolution has proven to be a highly versatile and successful tool for protein engineering.⁵⁻⁸ However, finding improved enzymes is often technically difficult or time consuming due to the large number of variants that must be screened. In the case of NAAAR this is especially true; the substrate and product are enantiomers which makes assay development difficult. To overcome this, we have generated an enantioselective genetic selection system that allows for high-throughput screening of mutagenic NAAAR libraries.

NAAAR activity has been detected in *Streptomyces atratus* Y-53, many *Amycolatopsis* species and more recently in several thermophiles.⁹⁻¹⁴ In these bacteria, NAAAR is believed to act as a bi-

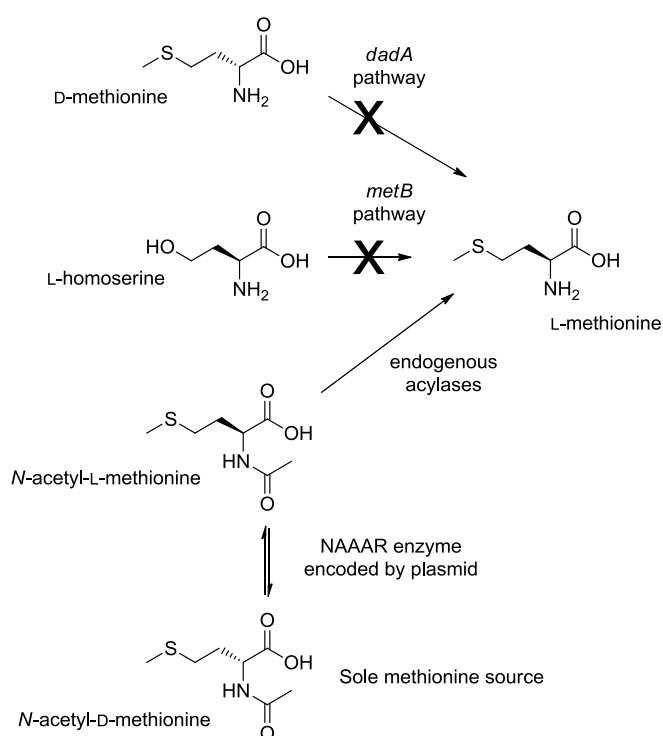
functional enzyme. Firstly, as an *o*-succinyl benzoate synthase (OSBS) that catalyses the formation of *o*-succinylbenzoate (a precursor of menaquinone) from 2-hydroxy-6-succinyl-2,4-cyclohexadiene carboxylate (Scheme 1c)¹⁵, and secondly as the racemase in a three-step D-amino acid detoxification pathway. This pathway is suggested to involve a D-specific *N*-succinyl transferase, an *N*-succinyl-amino acid racemase (Scheme 1d), followed by a L-specific *N*-succinyl amino acid hydrolase which together racemise D-amino acids.¹⁶ NAAAR has been well studied as a member of the enolase superfamily, and enzymatic analysis has shown that the NAAAR reaction proceeds *via* a divalent cation-dependent, enolate-based proton transfer catalysed by two lysine residues.¹⁷ The racemization of *N*-succinyl amino acids (and the OSBS reaction) is known to be ~20 fold-faster than with the equivalent *N*-acetylated amino acids. As the present acylase process for the production of enantiopure α -amino acids requires *N*-acetylated substrates, high activity towards these amino acid derivatives is essential. If a NAAAR variant could be isolated with sufficiently high activity, the current acylase KR process could be run as a coupled NAAAR/acylase DKR which would almost double the final yield of product.



Scheme 1. a) A D-acylase-based resolution of an *N*-acetyl-DL-amino acid to yield an enantiopure D-amino acid; b) a NAAAR/acylase coupled dynamic kinetic resolution. The two natural reactions of the OSBS/NAAAR protein; c) as an OSBS in the menaquinone pathway, d) as an *N*-succinyl amino acid racemase.

Enantioselective selection method

To efficiently screen mutagenic NAAAR libraries, a selection method was required that linked the rate of racemisation of an *N*-acetyl amino acid to the viability of an *Escherichia coli* (*E. coli*) host. This has been achieved by disabling the natural *E. coli* L-methionine biosynthetic pathway, and at the same time removing a D-amino acid racemization pathway (Scheme 2). Conversion of L-homoserine to L-methionine was abolished by deletion of the second enzyme in the L-methionine biosynthetic pathway, cystathionine γ -synthase (*metB*), while D-amino acid racemization was prevented by deletion of a non-specific D-amino acid dehydrogenase (*dadA*). DadA combines with an aminotransferase to racemise a variety of amino acids *via* their pro-chiral keto acid allowing toxic D-amino acids to act as a source of useful L-amino acids. As a result of these deletions, when grown in minimal media containing *N*-acetyl-D-methionine as the sole methionine source, the selection host is now dependent on recombinant NAAAR activity to racemise this to *N*-acetyl-L-methionine. An endogenous hydrolase then provides the source of L-methionine from the *N*-acetylated substrate. Using the λ -red mediated gene replacement/deletion method, *dadA* was deleted and *metB* replaced with a chloramphenicol resistance gene.¹⁸ This double-knock out host, named SET21, is now completely unable to synthesize L-methionine or racemise D-methionine. Importantly, the SET21 host is viable on *N*-acetyl-L-methionine allowing for a NAAAR-dependent screening method shown in Scheme 2. The ability of NAAAR to complement the SET21 strain and produce viable colonies was confirmed by transformation of SET21 with a plasmid expressing NAAAR wild type (WT) and selection on minimal media supplemented with *N*-acetyl-D-methionine as the sole methionine source (see supporting information).



← **Scheme 2.** Disabled L-methionine biosynthesis in *E. coli* SET21 host and NAAAR-dependent growth on *N*-acetyl-D-methionine.

Directed evolution

Using the SET21 selection host, mutagenic libraries of the *Amycolatopsis* sp. Ts-1-60 NAAAR gene were screened and the best variant used as the template for the next round of mutagenesis/selection. Variants were generated using XL1-Red ($>10^7$ variants), error prone PCR ($>10^5$ variants) and saturation mutagenesis ($>10^2$ variants). Active variants were selected by SET21 colony size when grown on minimal media supplemented with *N*-acetyl-D-methionine or by high throughput HPLC screening. Selection of libraries generated by XL1-Red mutagenesis highlighted the G291E mutation, which was subsequently improved upon by saturation mutagenesis to generate G291D. Error prone PCR on this clone generated NAAAR G291D F323Y which could not be improved upon after saturation mutagenesis of F323Y and a further round of error prone PCR. Saturation mutagenesis of other residues (D316 and S135) within the active site of NAAAR G291D F323Y failed to generate improved variants.

The NAAAR activity of each variant was determined by expression and purification of recombinant protein followed by kinetic analysis with a range of amino acids. The rate of racemisation was monitored with chiral HPLC and the activities of the best variant from each round of selection are shown in Table 1. Gratifyingly, the NAAAR G291D F323Y variant displayed the most improved activity with each of the substrates tested, showing ~2- to 6-fold improved K_{cat} values. The kinetic data would suggest that the mutations are directly affecting turnover and not binding as no major change in K_m was observed.

variant	<i>N</i> -acetylated amino acid substrate							
	L-methionine		D-methionine		D-(4-fluoro)phenylglycine		D-allylglycine	
	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)*
WT	20	18	14	40	41	35	97	N.D
G291E	48	40	27	40	103	35	125	N.D
G291D	80	40	54	40	125	35	153	N.D
G291D F323Y	95	40	67	50	272	40	232	N.D

Table 1. Whole cell biotransformations of NAAAR variants with various *N*-acetylated amino acids.

NAAAR/acylase DKRs

To measure the improvement in the DKR of *N*-acetyl-DL-amino acids with these variant NAAARs, small scale resolutions were performed using whole cell biocatalysts expressing an L-acylase and one

of NAAAR WT, G291E, G291D, or G291D F323Y. Combinations of the acylase and NAAAR hosts were mixed, and the resolution of *N*-acetyl-DL-methionine to L-methionine monitored over five hours by chiral HPLC (Figure 1). As expected, with no NAAAR present, *N*-acetyl-D-methionine showed no conversion to free L-methionine (data not shown). When BL21(DE3) cells expressing NAAAR WT were used, *N*-acetyl-D-methionine was now a substrate, *via* racemisation, for the L-specific acylase, however, the rate of racemization (k_{rac}) was clearly slower than the rate of hydrolysis (k_{hyd}) (Figure 1a). Similar results, with improved k_{rac} values were observed for both G291 variants; however k_{rac} was still slower than k_{hyd} (data not shown). Encouragingly, in reactions containing BL21(DE3) expressing NAAAR G291D F323Y, both enantiomers of *N*-acetyl-methionine were being consumed at the same rate, *i.e.* $k_{\text{rac}} \geq k_{\text{hyd}}$ (Figure 1b). This suggests that with NAAAR G291D F323Y, the process runs as an effective DKR. The ee of the final L-methionine was >99% (by chiral HPLC).

The performance of NAAAR G291D F323Y under process-like conditions was further investigated with a DKR of *N*-acetyl-DL-allylglycine using a partially purified NAAAR G291D F323Y biocatalyst generated from an *E. coli* fermentation (see Supporting Information). A coupled NAAAR G291D F323Y/D-acylase process was used to resolve 1g *N*-acetyl-DL-allylglycine to D-allylglycine with >89% yield (by isolated product) in 18 hours. Greater than 98% conversion was recorded by HPLC trace. The equivalent D-acylase kinetic resolution would give only a theoretical maximum of 50% conversion. Similar results were observed for other amino acids (data not shown). The low enzyme loading required and excellent yields suggest these processes would be economical at scale.

NAAAR G291D F323Y structure

Analysis of the wild-type NAAAR:*N*-acetyl-methionine structure (PDB code: 1SJA) revealed that both G291 and F323 are found within the *N*-acyl binding pocket, 5-6 Å from the substrate acyl group.¹⁹ To gain an insight into the molecular basis for the improvement in catalysis, the X-ray crystal structure of NAAAR G291D F323Y (PDB code: 4A6G) was determined. A dataset to a resolution of 2.71 Å was collected, and the structure solved using 1SJA as a model (see supporting information). Two novel interactions were observed that could be attributed to the G291D and F323Y mutations (Figure 2a). These are a salt bridge between the carboxylate of D291 and the guanidinium of R299 (2.7 Å), and an H-bonding interaction between the carboxylate of D291, and the hydroxyl of Y323 (2.8 Å). The resolution of the structure prevented identification of definitive changes in protein-ligand interactions; however, the ligand was slightly displaced compared to the published WT structure, suggesting that its binding environment had been altered.

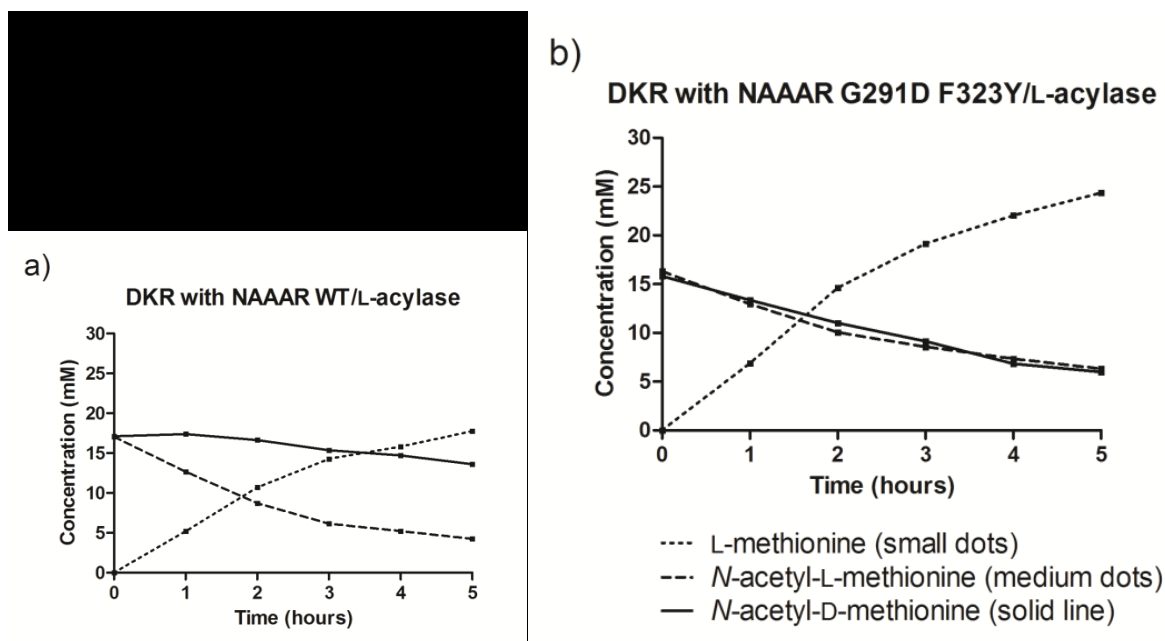


Figure 1. DKR of *N*-acetyl-DL-methionine by the coupled NAAAR/L-acylase process with a) NAAAR WT and b) NAAAR G291D F323Y. The decrease in *N*-acetyl-D-methionine concentration gives a measure of racemase activity (k_{rac}), and the drop in *N*-acetyl-L-methionine gives a measure of acylase activity (k_{hyd}). No major increase in L-methionine yield is observed as the reaction has not gone to completion and is limited by L-acylase turnover.

The most interesting observation can be made when comparing the structures of NAAAR WT complexed with *N*-succinyl-methionine (PDB code: 1SJC)¹⁹ and NAAAR G291D F323Y complexed with *N*-acetyl-methionine. Here, the carboxylate of G291D can be seen to be mimicking the succinyl carboxylate which is not present on the acetyl group. As NAAAR WT is 10-20 fold more active with *N*-succinyl amino acids than the equivalent *N*-acetyl substrates, it appears this interaction is of key importance for efficient turnover. In the NAAAR WT:*N*-succinyl methionine structure this interaction is made *via* two structural water molecules (Figure 2b). These observations are complemented by kinetic data that suggest that protein:ligand binding has not been dramatically affected with introduction of the G291D and F323Y mutations.

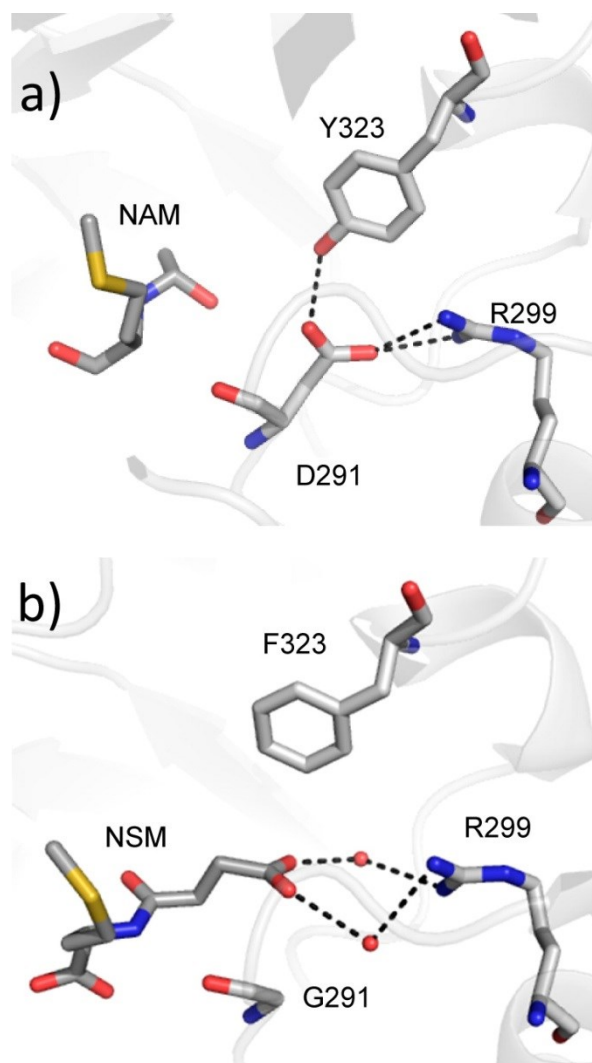


Figure 2. a) New interactions observed in the NAAAR G291D F323Y acyl binding pocket, NAM = *N*-acetyl-methionine. b) Interactions between succinyl carboxylate and R299 *via* two water molecules (red spheres), NSM = *N*-succinyl-methionine.

In summary, a variant NAAAR with increased activity towards a variety of synthetically useful *N*-acetyl amino acids has been evolved that shows promise as an industrial biocatalyst. When coupled with a stereo-selective acylase, gram level resolutions with excellent yields could be achieved within 18h at 50 g L⁻¹ substrate concentrations. Initial results would suggest these processes will be cost effective at scale for the production of a range of both proteinogenic and non-proteinogenic α -amino acids.

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