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Rational engineering of *Luminiphilus sylvensis* (*R*)-selective amine transaminase for the acceptance of bulky substrates†

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Despite the plethora of information on (*S*)-selective amine transaminases, the (*R*)-selective ones are still not well-studied; only a few structures are known to date, and their substrate scope is limited, apart from a few stellar works in the field. Herein, the structure of *Luminiphilus sylvensis* (*R*)-selective amine transaminase is elucidated to facilitate engineering towards variants active on bulkier substrates. The V37A variant exhibited increased activity towards 1-phenylpropylamine and to activity against 1-butylamine. In contrast, the S248 and T249 positions, located on the β -turn in the P-pocket, seem crucial for maintaining the activity of the enzyme.

Transaminases (TAs, E.C. 2.6.1.x) have attracted the scientific interest of the field due to their ability to easily synthesize optically pure amines.¹ The structure of the substrate-binding site, typically composed of a small and a large binding pocket over pyridoxal 5'-phosphate (PLP),² leads to excellent enantioselectivity in most cases. To expand the synthetic potential of these enzymes, several research groups have been working on the enlargement of the small binding pocket, which typically accommodates only a methyl group. There have been several cases of successful engineering of (*S*)-selective amine transaminases (ATAs), which belong to fold class I of PLP-dependent enzymes.³ The transfer of knowledge among (*S*)-ATAs has been proven feasible, and methodologies to identify (*S*)-selective ATAs active against bulky substrates from the gene pool have been proposed.⁴ However, the (*R*)-selective ATAs, which belong to fold-class IV, are less studied. The first methodology to guide (*R*)-ATA identification from the sequence pool was published in 2010 by Höhne and his coworkers.² In the same year, Savile and coworkers published stellar protein engineering work to

construct (*R*)-ATAs for the production of sitagliptin,⁵ highlighting their synthetic potential and evolvability. Since then, knowledge about (*R*)-ATAs is sparse; only a few structures are available, and we are still far from predicting the substrate scope of an enzyme solely from the sequence. Very recently, an interesting meta-analysis of the available structures of fold type IV TAs and their sequences shed some light on their substrate specificity.⁶

To contribute to this field, we decided to work with the (*R*)-selective ATA from *Luminiphilus sylvensis* (LS_ATA), which was identified by Höhne *et al.*, an enzyme previously mentioned as *Gamma proteobacterium* ATA.² This TA was selected, as it exhibited the highest activity against the benchmark substrate, 1-phenylethylamine (PEA), among several (*R*)-ATAs (data not shown), and its sequence is quite unique compared with the known fold IV enzymes. In public databases, LS_ATA is annotated as a “branched-chain amino acid transaminase” (BCAT). The closest resolved structure is the BCAT from the thermophilic archaea *Geoglobus acetivorans* (PDB code: 5cm0),⁷ which presents only 41% identity to LS_ATA. However, Höhne *et al.* already observed that the activity for valine synthesis was lower than that for PEA,² while BCATs exhibit the opposite catalytic profile.⁸ Höhne and coworkers suggested two motifs to distinguish BCATs from (*R*)-ATAs,² but LS_ATA does not have any of them. The β X- and β Y-strands of LS_ATA (GVFDVVSAW and ASIRFIVT, respectively) are a mix of the motifs found in BCATs and (*R*)-ATAs. However, the β -turn of the P-pocket of LS_ATA has the sequence STAG, which resembles more the motif of (*R*)-ATAs.⁶ Thus, as LS_ATA is structurally and functionally closer to the (*R*)-ATA, we will refer to it as such.

To guide our engineering efforts, we crystallized LS_ATA and determined its X-ray structure. The *L. sylvensis* (*R*)-ATA was structurally characterized at a resolution of 1.6 Å, in the orthorhombic space group $P2_12_12_1$, in the internal aldimine state between the PLP and catalytic lysine, K154. LS_ATA was found as a homohexamer built up of a trimer of dimers (Fig. 1). Gel filtration and blue native polyacrylamide gel electrophoresis

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Fig. 1 The hexameric form of LS_ATA (upper), where each chain is presented with another color, and the dimer (lower) where the internal aldimine (sticks) is formed in the interface. PDB code: 7p3t.

experiments (Fig. S1, ESI[†]) confirmed the homohexameric state. Although the dimeric structure is the most common for (*S*)-ATAs, several (*R*)-ATAs are organized in higher multimers. The TAs from *Geoglobus acetivorans* and *Archeoglobus fulgidus* also form

hexamers,⁷ while the (*R*)-ATA from *Thermomyces stellatus* was mainly found as the tetramer (~90%).⁹ A higher order of the self-organization of (*R*)-ATAs seems to be a common feature for stabilizing the functional dimer. PLP is bound in a canonical manner into the interdomain cleft, covalently linked with K154. The substrate binding site positioned between two monomers of the dimer is in an open conformation as the O-pocket loop (100–107) is highly flexible and directed into the bulk solvent. This finding is in contrast to that of *G. acetivorans* ATA in the complex with α -ketoglutarate (PDB code: 5e25), in which the O-pocket loop points towards PLP, and largely shields the substrate.⁷ Peisach and coworkers suggested that a “carboxylate trap” (a Tyr, an Arg, and a His residue) is responsible for the specificity of D-amino acid aminotransferases (DAATs).¹⁰ However, these residues are not found on the respective positions in LS_ATA.¹⁰

When LS_ATA is characterized with the two optically pure amines separately *via* a photometric assay,¹¹ it exhibits minor activity with (*S*)-PEA, especially at higher pH values (Table S1, ESI[†]). However, its preference for the (*R*)-amine was higher ($E_{app} \sim 70$), as expected.² To explain the enantioselectivity of LS_ATA, the structure was refined in water, and the lowest energy structure was selected for further analysis. The dimer of chains A and C had the best quality, according to Molprobit.¹² The quinonoids of (*R*)- and (*S*)-PEA were built in this dimer, as this is the most demanding intermediate of the catalytic mechanism.

As shown in Fig. 2, the quinonoid can be formed in both conformations. However, the Re-face quinonoid has a lower formation energy compared with the Si-face equivalent ($-1521.65 \text{ kJ mol}^{-1}$ and $-1497.861 \text{ kJ mol}^{-1}$, respectively), as the small binding pocket does not provide enough space for an aryl group. The selectivity for the (*R*)-enantiomer could also be attributed to the specific residues forming the binding pockets. The large binding pocket is formed from the polar residues F35, R92, and R158. These residues might be involved in stabilizing the aromatic ring of the Re-face quinonoid. F35 is at the correct



Fig. 2 The active site pocket of LS_ATA bearing the Re-face quinonoid (A) and Si-face quinonoid (B). The quinonoid intermediates are shown in green, and residues within 4 Å from the intermediate are shown in cyan (all belong to the A chain). The catalytic lysine, K154, is in front of the quinonoid. All non-polar hydrogens are removed for clarity.





Fig. 4 Conversion in the kinetic resolutions of (*R,S*)-PEA (circle) and (*R,S*)-PPA (triangle), as well as the deamination of (*R*)-PBA (square), using pyruvate as the amine acceptor from the LS_ATA wild-type (black) and the V37A variant (red). All experiments were performed at least in duplicate. Errors smaller than symbols are not presented. Experimental details are found in the ESI.†

was detected in the photometric assay. However, the activity is low, and even after 24 h (*R*)-PPA is not fully converted. The V37A mutation increases this activity, and the (*R*)-amine is fully converted after 24 h. This discrepancy with the data from the photometric assay led us to test the activity of both variants against (*R*)-PBA (4 mM). The V37A variant could convert (*R*)-PBA, with full conversion achieved after 24 h, while the wild-type did not exhibit any significant activity. The divergence between the two assays may arise from the substrate concentration difference, as the photometric assay was performed with 1 mM amine, while the kinetic resolution assay with 8 mM of racemate. The initial velocity observed for the wild-type with PPA is over the detection limit of the photometric assay. We hypothesize that the affinity constants for the amines are in the low mM range; thus the initial velocity is heavily influenced by the concentration.

The kinetic resolution mode is not synthetically interesting for (*R*)-ATAs, as they provide the (*S*)-enantiomer. There are several (*S*)-ATAs that can efficiently produce (*S*)-PBA.^{4,13b} Despite our efforts using several amine donors (isopropylamine, D-alanine, and (*R*)-PEA), we were not successful in asymmetric synthesis with propiophenone and butyrophenone (data not shown). It seems that the activity of the V37A variant is quite low to observe reasonable conversion in the asymmetric synthesis mode. We believe, however, that the V37A variant is a good template for protein engineering, to obtain efficient biocatalysts that can produce bulky (*R*)-amines.

Conclusively, the structure of LS_ATA, a unique enzyme – in terms of sequence – of fold class IV, will provide some insights into (*R*)-ATAs. We hope that our work will contribute to the understanding of the substrate scope of (*R*)-ATAs, and the guidance of protein engineering efforts. However, the mutations in the β -turn of the P-pocket showed that secondary

interactions are crucial for the activity of (*R*)-ATAs and that further studies on the role of the amino acids in the small binding pocket are required.

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Conflicts of interest

There are no conflicts to declare.

References

- (a) S. A. Kelly, S. Mix, T. S. Moody and B. F. Gilmore, *Appl. Microbiol. Biotechnol.*, 2020, **104**(11), 4781–4794; (b) M. D. Patil, G. Grogan, A. Bommarius and H. Yun, *Catalysts*, 2018, **8**(7), 254; (c) F. Guo and P. Berglund, *Green Chem.*, 2017, **19**(2), 333–360; (d) M. Fuchs, J. E. Farnberger and W. Kroutil, *Eur. J. Org. Chem.*, 2015, 6965–6982.
- M. Höhne, S. Schätzle, H. Jochens, K. Robins and U. T. Bornscheuer, *Nat. Chem. Biol.*, 2010, **6**, 807–813.
- (a) H. Land, F. Ruggieri, A. Szekrenyi, W.-D. Fessner and P. Berglund, *Adv. Synth. Catal.*, 2020, **362**, 812–821; (b) H.-G. Kim, S.-W. Han and J.-S. Shin, *Adv. Synth. Catal.*, 2019, **361**, 2594–2606; (c) M. S. Weiß, I. V. Pavlidis, P. Spurr, S. P. Hanlon, B. Wirz, H. Iding and U. T. Bornscheuer, *ChemBioChem*, 2017, **18**, 1022–1026; (d) M. Genz, O. Melse, S. Schmidt, C. Vickers, M. Dörr, T. van den Bergh, H.-J. Joosten and U. T. Bornscheuer, *ChemCatChem*, 2016, **8**, 3199–3202; (e) S.-W. Han, E.-S. Park, J.-Y. Dong and J.-S. Shin, *Appl. Environ. Microbiol.*, 2015, **81**, 6994–7002.
- I. V. Pavlidis, M. S. Weiß, M. Genz, P. Spurr, S. P. Hanlon, B. Wirz, H. Iding and U. T. Bornscheuer, *Nat. Chem.*, 2016, **8**(11), 1076–1082.
- C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman and G. J. Hughes, *Science*, 2010, **329**, 305–309.
- E. Y. Bezsudnova, V. O. Popov and K. M. Boyko, *Appl. Microbiol. Biotechnol.*, 2020, **104**, 2343–2357.
- M. N. Isupov, K. M. Boyko, J. M. Sutter, P. James, C. Sayer, M. Schmidt, P. Schönheit, A. Y. Nikolaeva, T. N. Stekhanova, A. V. Mardarov, N. V. Ravin, E. Y. Bezsudnova, V. O. Popov and J. A. Littlechild, *Front. Bioeng. Biotechnol.*, 2019, **7**, 7.
- E. Y. Bezsudnova, K. M. Boyko, A. Y. Nikolaeva, Y. S. Zeifman, T. V. Rakitina, D. A. Suplatov and V. O. Popov, *Biochimie*, 2019, **158**, 130–138.
- C. M. Heckmann, L. J. Gourlay, B. Dominguez and F. Paradisi, *Front. Bioeng. Biotechnol.*, 2020, **8**, 707.
- D. Peisach, D. M. Chipman, P. W. van Ophem, J. M. Manning and D. Ringe, *Biochemistry*, 1998, **37**, 4958–4967.
- S. Schätzle, M. Höhne, E. Redestad, K. Robins and U. T. Bornscheuer, *Anal. Chem.*, 2009, **81**(19), 8244–8248.
- I. W. Davis, A. Leaver-Fay, V. B. Chen, J. N. Block, G. J. Kapral, X. Wang, L. W. Murray, W. B. Arendall 3rd, J. Snoeyink, J. S. Richardson and D. C. Richardson, *Nucleic Acids Res.*, 2007, **35**, W375–W383.
- (a) Y. Xie, F. Xu, L. Yang, H. Liu, X. Xu, H. Wang and D. Wei, *Catal. Sci. Technol.*, 2021, **11**, 2461–2470; (b) A. Nobili, F. Steffen-Munsberg, H. Kohls, I. Trentin, C. Schulzke, M. Höhne and U. T. Bornscheuer, *ChemCatChem*, 2015, **7**(5), 757–760.
- M. Voss, C. Xiang, J. Esque, A. Nobili, M. J. Menke, I. André, M. Höhne and U. T. Bornscheuer, *ACS Chem. Biol.*, 2020, **15**, 416–424.

