Computational Redesign of an \( \omega \)-Transaminase from Pseudomonas jessenii for Asymmetric Synthesis of Enantiopure Bulky Amines

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ABSTRACT: \( \omega \)-Transaminases (\( \omega \)-TA) are attractive biocatalysts for the production of chiral amines from prochiral ketones via asymmetric synthesis. However, the substrate scope of \( \omega \)-TAs is usually limited due to steric hindrance at the active site pockets. We explored a protein engineering strategy using computational design to expand the substrate scope of an \( (S) \)-selective \( \omega \)-TA from Pseudomonas jessenii (PjTA-R6) toward the production of bulky amines. PjTA-R6 is attractive for use in applied biocatalysis due to its thermostability, tolerance to organic solvents, and acceptance of high concentrations of isopropylamine as amino donor. PjTA-R6 showed no detectable activity for the synthesis of six bicyclic or bulky amines targeted in this study. Six small libraries composed of 7−18 variants each were separately designed via computational methods and tested in the laboratory for ketone to amine conversion. In each library, the vast majority of the variants displayed the desired activity, and of the 40 different designs, 38 produced the target amine in good yield with >99% enantiomeric excess. This shows that the substrate scope and enantioselectivity of PjTA mutants could be predicted in silico with high accuracy. The single mutant W58G showed the best performance in the synthesis of five structurally similar bulky amines containing the indan and tetralin moieties. The best variant for the other bulky amine, 1-phenylbutylamine, was the triple mutant W58M + F86L + R417L, indicating that Trp58 is a key residue in the large binding pocket for PjTA-R6 redesign. Crystal structures of the two best variants confirmed the computationally predicted structures. The results show that computational design can be an efficient approach to rapidly expand the substrate scope of \( \omega \)-TAs to produce enantiopure bulky amines.

KEYWORDS: aminotransferase, substrate scope engineering, steric hindrance, green chemistry, computer-aided design, biocatalysis, protein engineering

INTRODUCTION

Transaminases are attractive biocatalysts for the synthesis of chiral amines, which are used as building blocks in the pharmaceutical industry. In the transamination reaction, an amino group is transferred from a donor (usually an amine or amino acid) to an acceptor (a ketone, keto acid, or aldehyde) with the enzyme acting as a molecular shuttle through its cofactor, the pyridoxal \( 5' \)-phosphate (PLP) group. The transaminases are very diverse, both in terms of the fold type and substrate range. The term \( \omega \)-transaminase (\( \omega \)-TA) is used for enzymes acting on aminoalkanoic acids with the amino and carboxylate groups on the opposite ends of an alkyl group. Most of the \( \omega \)-TAs also act as general amine transaminases and do not require a carboxylate group in the amino donor or acceptor. Accordingly, such enzymes can be used to convert ketones to the corresponding amines, which is of synthetic importance if the products are enantiopure.

The overall catalytic cycle of transaminases consists of two half-reactions, each composed of several reversible steps (Scheme 1). In the first half-reaction, the conserved lysine that forms a Schiff base with the PLP in the native protein (internal aldimine, E-PLP) is displaced by the amino donor, resulting in the formation of an external aldimine. Then, via formation of quinonoid and ketimine intermediates and imine hydrolysis, the amino group is transferred to PLP, forming the aminated cofactor pyridoxamine \( 5' \)-phosphate (PMP).
enzyme complex (E:PMP). The deaminated donor is released as a ketone, aldehyde, or keto acid. For the second half-reaction, the E:PMP enzyme binds the amino acceptor to regenerate the E-PLP via ketimine, quinonoid, and externalaldimine intermediates, after which the product is released as an amine or amino acid. In the case of non-symmetric ketones, ω-TAs convert the substrate to a chiral amine, offering an attractive catalytic route for the asymmetric synthesis of chiral amines.

As with most enzymes, the substrate scope of ω-TAs is rather restricted, often due to steric hindrance in the active site. Most ω-TAs are homodimeric fold-type I PLP proteins, with two identical active sites located at the interface of the monomers. Each active site of these class III transaminases has both a large and small binding pocket to accommodate substituents of the carbonyl (or amino-bound) central carbon atom. While the small binding pocket can seldom accommodate anything larger than a methyl group, the large binding pocket is generally capable of accepting an aryl or alkyl group. To expand the substrate range of ω-TAs, the binding pockets need to be engineered to accommodate different groups.

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variants with high conversion and high enantioselectivity toward four amines were found.15

The use of computational techniques in protein engineering is becoming an attractive option, in part due to the development of better energy functions and search algorithms.16 The caveat of using computational design is that the methodology is case-specific, and the choice of a good strategy is dependent on the enzyme family, the type of reaction, the type of substrate, and the limiting catalytic step. For ω-TAs, computational methods for finding mutations that modify the substrate scope typically include docking of a modeled reaction intermediate17 and molecular dynamics simulations17 or quantum mechanical modeling18 of such an intermediate. Docking is the fastest amongst these methods, but explicit water molecules are generally not included to avoid a drastic increase in the search space. Water may play an important role in both the catalytic mechanism and shaping the binding site of ω-TAs.19,20 Different reaction steps have been computationally modeled for the redesign of ω-TAs; hence, different ligands, that is, the ketone that acts as an amine acceptor21,22,23, the quinonoid intermediate,17,20,21 the amine product,17,20,21 the quinonoid intermediate,17,20,21 and the external aldimine intermediate24 have been examined. An advantage of using a reaction intermediate is that the search space for the conformation of the ligand is smaller since the positions of the PLP cofactor atoms can be considered fixed. However, working directly on a reaction intermediate implicitly assumes that the enzyme is capable of forming the Michaelis complex with the substrate (ketone or amine) and converting it to that intermediate and thus would neglect mutations that influence the entry of the substrates or their accommodation in a reactive orientation.

In computational predictions based on structural modeling, docking, and molecular dynamics simulations, mutations are usually chosen manually and investigated on a one-by-one basis, which seriously limits the accessible sequence space and complicates the prediction of variants with multiple substitutions. Alternatively, mutations can be generated in a single dock-and-design step from a user-defined search space. This way of discovering new enzyme variants without preselecting individual mutations is offered by design algorithms,25 as implemented in, for example, Rosetta enzyme design software.26 Often used for de novo protein and enzyme design, Rosetta can also be employed to modify the activity of existing enzymes.27 Rosetta uses a Monte Carlo search algorithm that randomly mutates selected positions and searches for low-energy solutions by varying residue identities, protein residue rotamers, and ligand conformations.29 Calculations are very fast, and the search space can be defined in terms of target positions, allowed mutations, and rotamer library density of target residues and ligands. Local backbone changes are allowed, and good numbers of primary designs carrying multiple mutations can be generated in a single dock-and-design step. The CASCO protocol employs Rosetta in combination with MD simulations for ranking.30 In the current work, we present a design strategy that uses Rosetta and employs the Rosetta interface energy as the main metric for the ranking of primary design mutants, obviating the need for more expensive MD simulations in the initial ranking phase. The strategy benefits from the target ligands having hydrophobic and rigid substituents;31 hydrophobic groups do not form hydrogen bonding networks involving water molecules, which are typically absent in docking approaches. Furthermore, we use docking with the target substrates covalently bound to the PLP in the form of the external aldimine intermediate, which greatly reduces the degrees of freedom of the binding poses and thereby the search space.

As the target enzyme, we have chosen a recently reported stabilized variant of the (S)-selective ω-TA from Pseudomonas jessenii (PjTA-R6, Temax 85 °C).32 The enzyme was engineered for increased stability by computational redesign of the subunit interface and catalyzed enantioselective production of (S)-1-phenylethylamine at high substrate concentration [100 mM acetophenone, 1 M isopropylamine (IPA)] and in the presence of 20% dimethyl sulfoxide (DMSO) at 56 °C. In view of the excellent performance of PjTA-R6 under these harsh reaction conditions, we considered PjTA-R6 to be a good template...
to accommodate the intended intermediates was evaluated by the Rosetta interface energy (docking score). The results showed that there is a correlation between the interface energy and the experimental yield, which can aid in narrowing down the search in future mutagenesis efforts. Experimental verification showed that the majority (97%) of the mutants gave better reactivity than the initial scaffold, which was not able to produce any of the six targeted amines. All mutants showed excellent enantioselectivity for the production of the (S)-amine.

Scheme 3. Schematic View of the Large and Small Binding Pockets of PjTA-R6 Bearing the External Aldimine Intermediate [PLP-Schiff Base with (S)-1-Phenethylamine] 46

“Target residues belonging to the large binding pocket are colored blue, and the target residue from the small binding pocket is colored red. K287 is the catalytic lysine

Materials and Methods

Computational Library Design. The crystal structure of PjTA-R6 (PDB 6TB1) served as a scaffold for docking of the target ligands. The scaffold was prepared by explicitly adding hydrogen atoms to the crystal structure of PjTA-R6 using an automatic Yasara procedure (the CleanAll and OptHydAll functions). All crystalized water molecules and ions were deleted from the structure. The modeled ligands are the external aldimine of 1a–6a, named, respectively, as 1EA – 6EA in this manuscript (Figure S1). Structures of 1EA–6EA were prepared by adding a covalent bond between the PLP C4’ atom and the corresponding amino substrate (1b–6b) to form the respective Schiff base. Geometry optimization of the resulting external aldime structures was performed with the semiempirical AM1 method using the COSMO implicit solvent model. Partial charges were derived using the AM1/BCC procedure. Rotamer libraries for ligands 1EA and 4EA–6EA were generated by random perturbation of non-hydrogen atom dihedrals (SampleDih Yasara routine) (Figure S1). Atoms originally belonging to the pyridoxal cofactor were kept fixed. Ligands 2EA and 3EA were represented by a single rotamer. The ligands (1EA–6EA) were initially aligned to the cofactor atoms originally found in the crystal structure. This initial conformation along with the rotamer library served as input for Rosetta design to generate the enzyme complexes containing the external aldime ligands. For this computational design, we used Rosetta build number 57,849, and for comparing experimental activities with interface energies, we used Rosetta build number 60,072, which outputs energies in kcal/mol. The following command line arguments were used for Rosetta docking: -enzedes, -cst_predock, -cst_design, -cut1 0.0 -cut2 0.0, -cut3 8.0, -cut4 10.0, -cst_min, -chi_min, -bb_min, -packing:use_input_sc, -packing:soft_rep_design, -design_min_cycles 3, -ex1:level 4, -ex2:level 4, -ex1aro:level 4, and -ex2aro:level 4. All residues within 10 Å of any ligand non-hydrogen atom were set to repackable, but residues 152, 225, 258, 119, 118, 324, 292, 59, 287, and 87 were set to NATRO (NATral ROtamer). Multiple runs were performed for each substrate, and varying combinations of residues were allowed to mutate (Table S1). For each run, 1000 decoys were generated, and the resulting mutants were ranked by their Rosetta interface energy. Figures representing Rosetta designs were created with VMD (https://www.ks.uiuc.edu/Research/vmd/).

In general, ranking mutants by the interface energy of the external aldime complex proved to be a good target function to guide mutagenesis (see the Results section). However, as an extra step to explain some outliers, the water displacement that would accompany substrate binding was estimated using MD simulations. These simulations were performed with enzyme–cofactor and enzyme–ligand complexes generated by Rosetta docking using GROMACS 2020 software and the AMBER99SB force field. Details are given in the Supporting Information.

Mutagenesis, Enzyme Expression, and Purification. Mutations were introduced in vector pET-20b (+)-His-PjTA-R6 via QuikChange mutagenesis and confirmed by DNA sequencing (Eurofins Genomics). PjTA variants were produced in Escherichia coli BL21(DE3) cultivated in 200 mL of Terrific Broth (TB) medium with 100 µg/mL ampicillin. Expression was induced with IPTG at OD600 0.6,
and cultivation was continued at 24 °C for 16 h. Cells were harvested by centrifugation, washed, and lysed by sonication in 20 mM potassium phosphate, pH 7.5, containing 500 mM NaCl and 20 μM PLP. Centrifugation (36,000×g, 45 min, 4 °C) gave cell-free extract, loaded on a 5 mL HisTrap column. Elution with a linear gradient of 0–500 mM imidazole in 20 mM potassium phosphate, pH 7.5, containing 500 mM NaCl and 20 μM PLP, followed by desalting with Econo-Pac 10DG columns (Bio-Rad) gave purified enzymes. Protein concentrations were determined with the Bradford assay, and purified enzymes were stored in aliquots at −80 °C in 50 mM potassium phosphate, pH 8.0, with 20 μM PLP.

**Thermal Shift Assays.** Apparent melting temperatures (T_m^app) were measured by a fluorescence-based thermal shift assay (ThermoFluo). Specifically, in the wells of an IQ 96-well PCR plate (Bio-Rad), enzyme samples of 20 μL (0.5 mg/mL protein in 50 mM potassium phosphate buffer, pH 8.0) were mixed with 5 μL of 50-fold diluted SYPRO Orange. The plates were sealed with the Microseal B adhesive sealer (Bio-Rad) and heated from 20 to 99 °C in a MyiQ real-time PCR machine (Bio-Rad) at a heating rate of 1 °C/min. The temperature at which the rate of fluorescence change (dT/df) was highest was taken as T_m^app. For determining stability under harsh conditions, enzyme samples for thermal shift assays were prepared in 50 mM phosphate buffer containing 1 M IPA, 20% DMSO, or both of these. The pH was set at 8.0.

**Reagents.** IPA, ketones (1a–6a), (S)-amines (1b–6b), and (R)-amines (1c–6c) (except 4b and 4c due to unavailability) (Figure S2) were purchased from Sigma-Aldrich. Synthesis of compounds 4b and 4c was attempted by redesigning PjTA-R6 variants; only a single enantiomer was obtained, and its identity was confirmed by gas chromatography mass spectrometry (GC–MS). PLP was purchased from Fisher Scientific. The purity of reagents was higher than 95%.

**Enzyme Reactions.** Transaminase-catalyzed amination reactions were performed at 1 mL scale in sealed glass vials at 56 °C using 50 mM potassium phosphate buffer, pH 8.0, containing 1 M IPA, 20 mM one of the ketones 1a–6a, 20% DMSO (or 2% DMSO in the case of 3a), 0.5 mM PLP, and 1 mg of purified enzyme. For analysis, 100 μL samples were quenched by adding 20 μL of 10 M NaOH, centrifuged, and extracted twice with 300 μL of ethyl acetate. The combined extracts were treated with anhydrous magnesium sulfate to remove water, and a total of 200 μL of each sample was used for GC analysis. Because of the instability of amines 4b, 5b, and 6b, they were derivatized to the acetamide before GC analysis. For this, 200 μL samples of the extracted products were mixed with 300 μL of acetic anhydride and 5 mg of 4-dimethylaminopyridine (DMAP). After 1 h reaction time (450 rpm, room temperature), samples were treated with 500 μL of water, and the organic layers were dried with anhydrous magnesium sulfate. The dried samples were used for GC analysis.

Kinetic parameters of transaminase reactions were determined by measuring depletion of aromatic ketones in the 96-well microplate format. The reaction mixtures (1 mL) contained 1 M IPA, varying concentrations (0–16 mM) of ketones (1a–6a), 0.05 mM PLP, and 0.05 mg/mL purified enzyme in 50 mM potassium phosphate buffer, pH 8.0. Reactions were performed at 56 °C and started by addition of the enzyme. At different times (1 min, 2 min, 4 min, and 8 min), 200 μL samples were quenched by adding 50 μL of 0.2 M NaOH. After centrifugation, 200 μL samples were transferred to a 96-well microplate, and the absorbance was measured in a plate reader. Samples from reaction mixtures with 2–16 mM ketone were diluted 2–16-fold before measurement. The depletion of ketones 1a, 2a, and 3a–6a was detected at 290, 310, and 320 nm, respectively, and initial rates were calculated using calibration curves (0–1 mM).

**GC Analysis.** The concentration and enantiopurity of amines 1b–6b were determined by chiral GC. Calibration was done with mixtures of racemic amines and the corresponding ketones (0.2, 2, and 20 mM) dissolved in the standard reaction system but without addition of the enzyme. Peaks for ketone, (S)-amine, and (R)-amine were identified with commercial standards. Because of the structural similarity to 5b and 6b, 4b was quantified using the same GC response curve. For details, see Supporting Information Figures S3–S9.

**Crystal Structures.** Before crystallization, PjTA variants W58G and W58M + F86L + R417L were further purified using a Superdex 200 13/300 column (GE Healthcare), equilibrated with 20 mM HEPES, pH 7.5, 100 mM NaCl, and 20 μM PLP. Proteins were concentrated to ~8 mg/mL using a Vivaspin Turbo 4 10K filter unit (Sartorius) and crystallized using hanging drop vapor diffusion in 24-well LINBRO plates (Molecular Dimensions Ltd). Per well, 1 μL of protein solution was mixed with 1 μL of reservoir solution containing 0.7–1.0 M sodium succinate, pH 7.6, as in our previous work on the thermostable parent PjTA-R6.32 Crystals of PjTA-R6 + W58G appeared after two days and grew to an average size of 0.4 × 0.3 × 0.3 mm³. Their yellow color indicated that PLP was bound. Large yellow crystals were also obtained for PjTA-R6 + W58M + F86L + R417L, with an average size of 0.3 × 0.2 × 0.1 mm³. Attempts to obtain structural information on the binding mode of the external aldimine included several soaking experiments with the amino donor IPA, the ketones 1a and 2a, or the amines 1b and 2b for variants PjTA-R6 + W58M + F86L + R417L and PjTA-R6 + W58G. X-ray diffraction data were collected at beamlines I24 and I04 at Diamond Light Source (Oxford, UK). The diffraction data were indexed and integrated using xia2/Dials and then scaled and merged with Aimless from the CCP4 software suite.35 The crystals of PjTA-R6 + W58G and PjTA-R6 + W58M + F86L + R417L belonged to the same space group as PjTA-R6, with nearly identical unit cell dimensions, allowing the PjTA-R6 structure (PDB 6TB1) to be used for initial refinement and electron density map calculations. Subsequently, the structures were adjusted by model building to replace the side chains of the mutated residues. After a few cycles of refinement with RelMac56 followed by model building and water placement with Coot, the structure was completed. A summary of data collection is provided in Table S3. Figures showing crystal structures were produced with PyMOL.36

**RESULTS**

**Computational Design of PjTA Variants.** To examine the use of a single dock-and-design step for shifting the substrate range of a transaminase toward acceptance of more bulky substrates, we selected the PjTA-R6 variant of the transaminase from *P. jessenii* as the template. PjTA-R6 is a thermostable variant (T_m^app = 85 °C) of the native 6-aminohexanoate transaminase (PjTA, T_m^app = 62 °C) of the caprolactam biodegradation pathway. PjTA-R6 contains six point mutations (P9A + E38Q + A60V + S87N + M128F + W58G) that increase its thermostability, of which positions 60, 87, and 154 are located near the active site. It does not accept

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Table 1. Mutant Dataset for the Asymmetric Synthesis of 1b–6b

| mutant | no. | target amines | Rosetta interface energy (kcal/mol) | $\Delta T_m^\text{ss}$ (°C) | yield (%) | ee (%) |
|--------|-----|---------------|------------------------------------|-----------------------------|-----------|--------|
| PjTA-R6 | 1   | 1b–6b         | 0                                  | n.a.                        | n.m.      |        |
| W58G   | 2   | 2b–6b         | −21.6/22.1/22.8/21.5               | 0                           | 51/64/69/69/69 | >99 |
| W58G + F86S | 3 | 1b, 3b, 4b | −21.8/22.1/21.7              | 8                          | 52/30/47            | >99 |
| W58G + F86L | 4  | 3b          | −21.9                          | +2                           | 27         | >99 |
| F86L + Y115F | 5  | 1b          | −23                            | −11                          | 38         | >99 |
| L57D + Y115F | 6  | 2b          | −21.6                         | +1                           | n.a.       | n.m.  |
| W58G + F86L + R417L | 7 | 1b–4b, 6b  | −21.7/21.4/22.3/21.3/21.6 | +3                          | 49/12/73/12/73 | >99 |
| W58M + F86L + R417L | 8 | 1b          | −22.9                         | −5                           | 72         | >99 |
| W58G + F86S + A230G | 9 | 1b          | −21.1                         | −2                           | 2          | >99 |
| W58G + F86S + R417L | 10 | 1b, 2b, 3b | −21.9/21.3/22.4            | +4.5                         | 31/6/27             | >99 |
| W58G + F86L + I261A | 11 | 1b, 4b      | −21.2/20.6                    | −8                           | 11/8       | >99 |
| W58G + F86N + R417L | 12 | 2b–6b       | −21.3/22.4/20.8/19.9/21.7    | −2                           | 73/62/12/32/73 | >99 |
| W58G + F86N + R417F | 13 | 3b–6b       | −22.4/21.4/20.2/21.7         | −3                           | 53/59/12/28/53 | >99 |
| W58G + F86L + R417F | 14 | 3b–6b       | −22.3/21.2/20.5/21.6         | −6.5                         | 47/70/17/20 | >99 |
| W58G + F86N + R417I | 15 | 4b          | −21                           | −5                           | 66         | >99 |
| W58G + F86L + R417I | 16 | 4b          | −20.8                         | −3                           | 69         | >99 |
| M54T + W58G + R417L | 17 | 4b          | −21.6                         | +2                           | 78         | >99 |
| M54T + W58G + R417I | 18 | 4b          | −21.7                         | +2                           | 57         | >99 |
| M54T + W58G + R417Q | 19 | 4b          | −21                           | −34                          | 1          | >99 |
| M54T + W58G + F86S | 20 | 5b          | −22.7                         | −8                           | 1          | >99 |
| M54T + W58G + F86L | 21 | 5b          | −22.4                         | −3                           | 2          | >99 |
| W58G + F86L + I261N | 22 | 5b          | −20.7                         | −11                          | 1          | >99 |
| W58G + F86C + A230P + R417L | 23 | 1b          | −21.6                         | 0                            | 37         | >99 |
| W58G + F86S + A230G + R417L | 24 | 1b          | −21.5                         | −1                           | 37         | >99 |
| W58G + F86N + A230G + R417L | 25 | 1b          | −21.5                         | −5.5                         | 28         | >99 |
| L57E + W58G + Y151C + R417K | 26 | 2b          | −21                          | −3.5                         | n.a.       | n.m.  |
| W58G + F86L + I261A + R417L | 27 | 1b, 2b     | −21.2/20.7                   | −7                           | 21/1       | >99 |
| W58G + F86L + I261V + R417L | 28 | 3b          | −22.1                         | −4                           | 37         | >99 |
| W58G + F86L + I261V + R417F | 29 | 3b          | −22.1                         | +1                           | 39         | >99 |
| W58G + F86L + I261A + R417F | 30 | 3b          | −21.5                         | +5.5                         | 14         | >99 |
| W58G + F86S + I261A + R417L | 31 | 3b          | −21.6                         | +6                           | 9          | >99 |
| M54T + W58G + F86S + R417L | 32 | 4b          | −20.8                         | −32                          | 15         | >99 |
| M54T + W58G + F86L + R417F | 33 | 4b          | −21                           | −33                          | 14         | >99 |
| W58G + F86L + A230P + I261V | 34 | 5b, 6b    | −20.8/19.1                   | −5                           | 5/4        | >99 |
| M54T + W58G + I261A + R417L | 35 | 5b          | −18.4                         | −1                           | 1          | >99 |
| W58G + F86L + A230P + I261V + R417L | 36 | 4b, 6b    | −20/19.9                     | −5                           | 20/12      | >99 |
| W58G + F86N + A230P + I261V + R417L | 37 | 4b, 6b    | −19.9/20                     | −2                           | 6/10       | >99 |
| W58G + F86L + A230P + I261V + R417T | 38 | 4b, 5b, 6b | −19.2/19.3/19.6              | −2                           | 14/4/4      | >99 |
| W58G + F86L + A230P + I261V + R417Q | 39 | 6b          | −21.1                         | 0                            | 11         | >99 |
| M54T + W58G + F86L + A230P + I261V + R417L | 40 | 4b, 6b    | −19.5/19.1                   | −2                           | 9/4        | >99 |
| M54S + W58G + F86L + A230P + I261V + R417L | 41 | 6b          | −19.1                         | −3                           | 5          | >99 |

The difference in $T_m^\text{ss}$ between PjTA-R6 and mutants. The $T_m^\text{ss}$ of PjTA-R6 is 85 °C. Interface energies were not determined since no catalytic poses were obtained. n.a. = no activity. n.m. = not measured. The mutant with the best analytic yield of the corresponding amine. Variants with poor stabilities.

For each ligand, 3–5 independent dock-and-design runs were performed, and in each run, 4–6 active site residues were allowed to mutate. The number of runs and the identity of the residues selected for mutagenesis were case-specific for each compound (Table S1).

The runs generated approximately 100–400 unique primary designs per ligand. These designs were ranked on the basis of their Rosetta interface energy, and the top 10–20 enzyme variants were selected. The resulting designed libraries contained mutations on positions Met54, Leu57, Trp58, Tyr151, Ala230, Ile261, and Arg417, belonging to the large binding pocket, and on position Phe86, belonging to the small binding pocket. Finally, 7–18 of the top-scoring mutants for each substrate were selected for experimental verification (Table 1). No enzyme variant was manually added to the

any of the ketones 1a–6a as substrates for transamination to 1b–6b, respectively.

Derivatives with an expanded substrate acceptance were predicted using Rosetta in a single dock-and-design step. Rosetta design calculations were performed for each of the substrates 1a–6a by optimizing the active site for binding of the corresponding external aldimine intermediates 1EA–6EA (Figure S1). Reshaping the binding pocket of PjTA-R6 was mainly aimed at alleviating steric hindrance caused by the bulky hydrophobic substituent on the carbonyl carbon of the substrate. Based on structural inspection of the crystallographic structure of the external aldimine formed from 6-amino-6-oxohexanoic acid by PjTA (PDB 6G4E), the search space comprised residues near the binding site (Table S1).
dataset. In total, 70 Rosetta designs were selected, which included 40 designs with different sequences because some designs for different substrates had the same sequence.

**Experimental Verification of Amine Synthesis.** All 40 unique designed mutants were constructed by site-directed mutagenesis. They expressed well in the soluble form in *E. coli* and were purified (Table 1). All but two of the mutant enzymes showed the desired catalytic activity and displayed high enantioselectivity (ee > 99%) for the production of chiral amines 1b–6b. Amine yields in these asymmetric transformation reactions varied from 1–86%, the latter value being similar to what was obtained with *Pj*TA-R6 in the synthesis of (S)-1-phenylethylamine from its preferred substrate acetophenone. Reactions were done with 1 M IPA in the presence of 20% DMSO, indicating that the robustness that was engineered previously into *Pj*TA-R6 was well maintained.

Gratifyingly, there was reasonable agreement between the docking results and yields in amine synthesis (Figure 1). In general, better Rosetta interface energies of the external aldimines 1EA–6EA correlated with higher yields in amination reactions. The best variant for the synthesis of amines 1b–6b either was the top-ranking variant (for compounds 2, 4, and 5) or occurred among the top-ranking variants (compounds 1, 3, and 6) based on the interface energy (Table 1). This indicates that the computed Rosetta interface energies can be helpful to distinguish the best-performing variants among the larger numbers of primary designs generated using the Rosetta search algorithm.

The trend between these docking scores and experimental yields was not followed by all redesigned *Pj*TA-R6 derivatives. A possible cause could be that underperforming variants are less stable and inactivated under the harsh conditions of the amination reactions (56 °C, 1 M IPA, 2–20% DMSO). Since the selection of designs for experimental verification was based on scoring of Rosetta interface energies and did not consider possible effects of mutations on stability, we examined the thermostability of all the designed *Pj*TA-R6 variants (Table 1). Only 3 out of 40 variants had substantially lower thermostability than *Pj*TA-R6 (mutants 19, 32, and 33; Δ*T*~m~ from −34 to −32 °C). Of the other 37 designs, 22 had a modest reduction of thermostability (Δ*T*~m~ from −2 to −11 °C), whereas 12 variants showed the same or slight increase in stability.

The loss of thermostability of variants 19, 32, and 33 was indeed accompanied by a detrimental effect on the yield of amine 4b under the harsh conditions of the reactions (56 °C, 1M IPA, 2–20% DMSO) in comparison to variants that were designed for the same product and showed higher stability (variants 15–18) (Table 1). Nevertheless, the three unstable variants still gave a higher yield of amine 4b than the parent *Pj*TA-R6 enzyme, which may be due to some initial activity, followed by inactivation under turnover conditions. Mutant 20 (∆*T*~m~ = −8 °C) also was less thermostable than the parent and gave a lower yield than expected from the trends shown in Figure 1 (5EA).

To further examine the role of enzyme stability under reaction conditions, three variants with different thermostabilities were used to test the effect of DMSO and/or IPA in thermal shift assays (i.e., variant 8, variant 19, and variant 20) (Table S2). Variant 8 (*T*~m~ = 80 °C), which followed the trend between yield and Rosetta interface energy (Figure 1, 4EA), had normal stability in the presence of DMSO or IPA. It was partially inactivated and showed reduced stability only after prolonged incubation under reaction conditions. The less-thermostable variant 19 (*T*~m~ = 51 °C) was partially unfolded and very sensitive to a high concentration of IPA or DMSO, as indicated by a further reduction of *T*~m~ (Table S2). Initial ketone conversion activities in the presence of IPA and DMSO were also determined, using a ketone concentration of 20 mM. According to the Rosetta interface energies, variant 19 was expected to be about half as active as variant 2, but its activity with ketone 4a in the presence of DMSO and IPA (0.06 U/
excellent yields in the production of stability. While mutants M54T + W58G + R417[L/I] showed mutation is responsible for the drastic reduction in thermostability, a possible cause of deviations from the relationship between interface energy and performance in synthesis is the occurrence of a structure unsuitable for catalysis (see below).

Causes and Effects of Reduced Thermostabilities. A closer inspection of mutants 32 and 33 revealed that a single mutation is responsible for the drastic reduction in thermostability. While mutants M54T + W58G + R417[L/I] showed excellent yields in the production of 4b, introducing mutation F86[L/S] substantially lowered amine production and decreased the $T_{m}^{app}$ by more than 30 °C. The mutations F86[L/S] were intended to alleviate steric clashes of Phe86 with the tetralin moiety (Figure S10). The structural models showed that Phe86 has π−π interactions with Tyr20 and Arg417. The latter in turn interacts with Asn87, which was previously introduced by an S87N substitution that enhances thermostability. Most other mutants carrying a substitution on position F86 showed a small reduction in thermostability [25 F86X mutants showed decreased thermostability, whereas six were more thermostable (cutoff of 1 °C)]. In variants 5 and 26, which maintained Phe86, mutation of Tyr151 led to a reduction in thermostability, which we attribute to the loss of an interaction via a water molecule with the phosphate of the PLP group (Figure S11). Overall, we found the template PjTA-R6 to be robust enough to accept the majority of the proposed mutations with no substantial decrease in $T_{m}^{app}$, but the effect of some of the mutations on the thermostability of the enzyme was difficult to predict. This especially holds for variants carrying multiple mutations that remove and create interactions that contribute to stability.

Figure 2. Designed binding sites for externalaldimes. (A–F) Rosetta-docked external aldimine complexes (orange) of enzyme variants PjTA-R6 + W58M + F86L + R417L for 1EA (A) and PjTA-R6 + W58G for 2EA–6EA (B–F). Amino acids of designed variants are colored in cyan (carbon), red (oxygen), and blue (nitrogen). Non-polar hydrogens are not shown, with the exception of hydrogen Hα that is, the hydrogen abstracted from the external aldimine (Figure S12). The superimposed crystal structure with the non-substituted residues of PjTA-R6 (magenta) is shown for comparison [i.e., Trp58, Arg417, and Phe86 in panel (A) and Trp58 in panels (B–F)]. Figures were rendered using VMD.

To examine if the observed activities of the Rosetta designs can be rationalized, we inspected the predicted structures (Figure 2). Variant PjTA-R6 + W58M + F86L + R417L was the best-performing mutant for the asymmetric synthesis of 1b, giving an amine yield of 72%. In this and other designs optimized for 1b, mutation of Trp58 to Met turned out to be better for accommodating 1EA than mutation W58G, which also occurred frequently (e.g., PjTA-R6 + W58G + F86L + R417L; 49% yield). While mutation W58G only eliminates steric hindrance, mutation W58M maintains a more hydrophobic binding site in addition to eliminating protein interactions that contribute to stability.

Rationalization of the Observed Activities. To examine causes of deviations from the relationship between interface energy and performance in synthesis is the occurrence of a structure unsuitable for catalysis (see below).

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Ketones containing the indane (2a) or tetralin (3a–6a) group required a PjTA-R6 variant with mutation W58G to function as an amino acceptor. In the external aldimine form, the aromatic ring of indane or tetralin must bind in the large binding pocket for production of the (S)-enantiomer of the amine. Unlike the aromatic ring in 1EA, the indane and tetralin bicyclic structures of 2EA–6EA allow no rotational mobility for the aromatic ring, causing steric hindrance with Trp58. The
WS8G single mutation was not enough to alleviate this steric hindrance, and the PjTA-R6 + WS8G mutant produced enantiopure (S)-amines 2b−6b in yields ranging from 29 to 86% (Table 1). Additional mutations on top of WS8G also gave active variants for conversion of ketones 2a−6a to amines but did not have a further beneficial effect, which was reflected in both the Rosetta interface energy and the experimental data. All variants carrying multiple mutations remained active and highly enantioselective.

From the experimentally tested mutant library of 40 unique variants (70 different enzyme−ligand combinations), only two variants did not exhibit catalytic activity toward the intended compound. The LS7D + Y151F (mutant 6) and LS7E + WS8G + Y151F + R417K (mutant 26) derivatives of PjAT-R6 were inactive on 2a (Figure 1), and neither was capable of accommodating 2EA in a catalytic pose, as confirmed by visual inspection of the designed structure (Figure S12). In variant PjTA-R6 + LS7D + Y151F, the aromatic ring of the bicyclic indane group of 2EA cannot be accommodated due to steric clashes with Trp58, which the mutations LS7D + Y151F did not alleviate. Thus, no catalytic pose was obtained in the docking procedure, rendering the calculated Rosetta interface energy for this mutant irrelevant for predicting catalytic potential. Furthermore, mutation LS7D introduced a hydrogen bond between the side chains of Asp57 and Thr324, which might cause the mutant to be inactive even if the steric clashes with Trp58 were to be alleviated. Residue Thr324 is believed to assist Lys287 in catalysis by forming a hydrogen bonding network.19 Glu57 in variant PjTA-R6 + LS7E + WS8G + Y151F + R417K can be disruptive for the very same reasons. Additionally, the formation of a salt bridge between Glu57 and Lys417 may prevent free movement of Glu57 upon substrate binding, causing steric clashes with the indane moiety, rendering the enzyme inactive (Figure S12). The steric clashes were reflected in the poor interface energy of mutant PjTA-R6 + LS7E + WS8G + Y151F + R417K (Table 1 and Figure 1). Thus, for the two designs, we propose a combination of steric hindrance and disruption of catalytic conformations as the cause of inactivity in the production of 2b.

The computational framework also yielded PjTA-R6 mutants suitable for the production of 3b. Due to the bicycle rigidity, the aromatic ring of the tetralin moiety has steric clashes with Trp58 of PjTA-R6 (Figure 2), which were eliminated by mutation WS8G. The tetralin differs from the native substrate 6-aminohepxanoic acid in that the latter is flexible and points outward into the tunnel where it forms a salt bridge with Arg417, thus avoiding steric clashes with Trp58 (Figure S13). In fact, the best variant for the production of 3b was the single mutant PjTA-R6 + WS8G, and additional mutations did not improve the yield. Furthermore, a design run in which Trp58 was not allowed to mutate did not result in designs with a good docking score (Table S1).

The different positions of the methoxy group attached to the tetralin moiety in intermediates 4EA, 5EA, and 6EA created distinctive trends. Most of the mutants designed to accommodate 4EA showed higher yields than designs for 5EA or 6EA. This difference can be rationalized by the position of the methoxy group in the tetralin moiety. The 5-methoxy substituent on the phenyl group of 4EA points toward the binding site entrance, making it easier to fit 4EA in the large pocket (Figures 2D and S14) than 5EA or 6EA. In other variants, the position of the methoxy group could act in favor of 6EA; the mutation R417Q was found to be beneficial for accommodating 6EA, increasing the product yield from 4% (mutant 34) to 11% (mutant 39) (Table 1). The side chain −NH2 of Gln417 can form a hydrogen bond with the oxygen lone pairs of −OMe (O−H distance of 2.0 Å) in the case of 6EA, but such a hydrogen bond would not form with 4EA as the −OMe is too far away (>5 Å) (Figure S14B). Accordingly, in the series MS4T + WS8G + R417K, where X = L, I, and Q, the product yields decreased from 78 to 1% (Table 1). It must be noted that the abovementioned rationalization only considers protein−ligand binding interactions of catalytic conformations of the complex but not the intrinsic ligand reactivities as the cause for differences in the observed reaction yields.

**Effect of Mutations on the Hydrophobicity of the Binding Site.** As mentioned above, most mutants with better Rosetta interface energy scores were better at converting ketones 1a−6a to the corresponding amines, and some variants deviating from this trend had a reduced thermostability. To explain a few outliers that do not have a decreased thermostability, that is, red circles in the 3EA, 4EA, or 5EA datasets (Figure 1), we examined if the number of water molecules that would need to be displaced for the tetralin moiety to fit in the binding pocket could play a role (Figure S15). A large displacement of water molecules may significantly impact the binding energy of ligands.31,32 We used ns-scale MD simulations to quantify the difference in the water-accessible volumes of the Rosetta-generated structures (Figure S16). Although this approach did not explain the outliers of 3EA, 4EA, or 5EA, when considering all variants (outliers or not), we did see a weak trend in the plot of the average difference in the number of water molecules between the enzyme−PMP complex and the enzyme−external aldimine complex against the experimental yield (Figure S17). The trend is weak, but it might be worth considering in further studies because water displacement upon substrate binding may contribute to a larger energy barrier that is not accounted for in the Rosetta calculations.33

**Crystal Structures Confirm Local Changes that Create Space.** To further examine the structural basis of the activity with bulky amines and the accuracy of the Rosetta predictions, we determined crystal structures of the PjTA-R6 + WS8G and PjTA-R6 + WS8M + F86L + R417L variants. Crystallization conditions were as reported earlier.32 Only structures with the PLP- or PMP-bound enzyme were obtained; attempts to obtain structures of PjTA-R6 variants with bound external aldimines by briefly soaking crystals with substrates failed. The crystal structures for the two best variants were refined to 1.7 Å resolution with an R-factor of 0.173 (Rfree = 0.191) for PjTA-R6 + WS8G and to 1.9 Å resolution with an R-factor of 0.177 (Rfree = 0.207) for PjTA-R6 + WS8M + F86L + R417L (Table S3).

The PjTA-R6 + WS8G structure contains the PLP cofactor as internal aldimine with PLP covalently linked to Lys287, while the PjTA-R6 + WS8M + F86L + R417L structure contains PMP as a result of the soaking with 1b. Similar to what was found in previous work,13 the inserted mutations did not affect the overall backbone conformation of these PjTA-R6 mutants in comparison to the PjTA-R6 structure. All mutated residues showed well-defined electron density, allowing unambiguous assignment of their side chain conformations. The PjTA-R6-WS8G crystal structure was in agreement with the Rosetta-predicted structure (RMSD = 0.31 Å) and showed
no deviations from the PyTA-R6 structure beyond the affected residues (Figure 3). Residue Trp58 is located deep in each subunit, above Lys287 and the PLP cofactor, and participates in the formation of the large binding pocket. By enlarging this pocket, mutation W58G contributes to an increase in the active site volume from 669 Å³ in PyTA-R6 to 768 Å³ in the PyTA-R6 + W58G variant, as calculated with PyVOL (PLP omitted). This reduces the steric hindrance that interferes with the binding of bulky aromatic substrates. The mutation thus facilitates accommodation of the bicyclic structure of the external aldimine formed with compound 3a.

The PyTA-R6 + W58M + F86L + R417L structure is also in agreement with the Rosetta prediction (RMSD = 0.32 Å), with negligible differences in side chain orientations (not shown). The mutations increased the active site volume (PLP omitted) from 669 Å³ to 1187 Å³. Superimposition of the crystal structure with Rosetta-docked poses for 1EA showed that the substrate’s phenyl ring is oriented toward the additional space in the large binding pocket obtained by replacement of Trp58 with a methionine. The propyl group is placed in the small binding pocket, which shows a slight increase in volume due to mutation F86L (Figure 4C). Mutation R417L further increases the active site volume by removing a constriction to the large binding pocket (Figure 4D). Thus, the W58M, F86L, and R417L mutations reshape the active site and reduce steric hindrance, which is necessary for accommodating intermediate 1EA and enables amine synthesis.

Yields in Amine Synthesis. In agreement with the purpose of the computational design work, the designed mutants gave higher yields of amines 1b−6b in IPA-driven transamination reactions than PyTA-R6. However, yields were still modest in most cases. For example, only up to 29% yield was achieved in the synthesis of 5b (Table 1). Reaction progress curves showed that under standard conditions (Scheme 2), accumulation of amine in the reaction mixtures did not increase after 24 h even for the best substrate−mutant combinations (Figure 5A). Furthermore, even over the first 1−4 h reaction time, amination activities were lower than expected from kinetic parameters determined under initial rate conditions (Table 2).

To identify the cause of partial conversion and low reaction rates and to find possible remedies, different measures were examined. We tested if improvement of yield could be achieved by extending reaction times after addition of fresh enzyme (1 mg), PLP (0.5 mM) or IPA (0.5 M). Also doubling the amount of enzyme added at the beginning and removing acetone by performing the reactions under low air pressure (40 kPa) during the first 24 h incubation time were attempted. However, neither of these measures had an effect on the yield.

The pH of the reaction mixtures remained constant. In contrast, when ketones (20 mM) were added again after 24 h reaction time, production of amines continued with all six substrates (Figure 5B, Table 2). The increase in amine product concentration after ketone addition suggests that the 24 h reactions had reached thermodynamic equilibrium. To examine this, the apparent reaction quotient (Kq) was calculated at 24 h and 48 h by eq 1.
The aim of this work was to explore the use of computational design with the Rosetta search algorithm for broadening the substrate scope of a transaminase. For this, we used PjTA-R6 as the template because its engineered stability should result in high mutability.\(^1\) The protocol avoided multiple iterations and manual selection of mutations by employing Rosetta enzyme design to obtain, in a single step, variants that catalyze amination of structurally demanding ketones. The presented methodology for broadening the substrate scope showed a high hit rate (68 out of 70 designs were successful) and good predictive power (Figure 1). The screening framework is computationally inexpensive (~2 CPU hours per enzyme variant using the HP workstation Z4 with an Intel Xeon W-2135 processor) and can easily be parallelized using multiple cores for high-throughput screening. Another attractive feature of the protocol is that combinations of mutations can be found using the Rosetta search algorithm, without the need to preselect them by rational inspection; only the search space needs to be defined. Other useful methodologies for predicting the catalytic potential of \(\omega\)-TA variants (using a different intermediate) are computationally more expensive (e.g., \(\sim 60\) CPU hours per enzyme variant in the methodology described by Voss et al.\(^17\)) or make use of coarser categorizations such as active vs inactive in the work of Sirin et al.\(^18\) and high- vs low-reactivity grouping used by Han et al.\(^21\) A study in which the activity toward 2-acetylbiphenyl ketone was engineered in \(\psi\)TA followed an approach in which mutations were added in a stepwise manner, without predicting activity for a large number of variants as a ranking tool.\(^20\)

We chose the external aldimine as the ligand for the docking calculations because its conversion to the geminal diamine intermediate in the amine synthesis half-reaction involves a high-energy transition state.\(^19\) Thus, we hypothesized that mutations that increase the stability of the external aldimine complex would have an observable effect on product formation.\(^17\) An added advantage of using a ligand covalently bound to a rigid cofactor is that the position of the reactive atom (\(C_{\alpha}\)) is known beforehand, which reduces both the docking search space and the number of variants that need to be screened to find a good mutant. Although the methodology described in this paper shows promising results for engineering the \(\omega\)-TA activity, special attention must be paid to the identity of enzymes that can be screened to obtain a higher yield at 48 h (%)

\[
K_{Q} = \frac{[\text{Am}] \times [\text{Ac}]}{[\text{K}] \times [\text{IPA}]}
\]

here, \([\text{Am}]\) and \([\text{Ac}]\) are the concentrations of amine and acetone, respectively, and \([\text{K}]\) and \([\text{IPA}]\) are the remaining concentrations of ketone and IPA, respectively. \([\text{IPA}]_0\) and \([\text{K}]_0\) are the added concentrations of IPA and ketones, respectively. \(K_{Q}\) values were calculated assuming \([\text{Ac}] = [\text{Am}], [\text{IPA}] = [\text{IPA}]_0 - [\text{Am}],\) and \([\text{K}] = [\text{K}]_0 - [\text{Am}].\) Since \(K_{Q}\) values at 24 h were higher than at 48 h (Table 2), only the former can represent equilibrium. Possibly, the enzyme loses activity in the 24–48 h interval, where the enzyme is exposed to a higher concentration (>20 mM) of the substrate. The results suggest that the 24 h yields reported in Table 1 for each substrate are not limited by catalytic activity but by the reaction mixtures approaching thermodynamic equilibrium.

**DISCUSSION**

The aim of this work was to explore the use of computational design with the Rosetta search algorithm for broadening the substrate scope of a transaminase. For this, we used PjTA-R6 as the template because its engineered stability should result in high mutability.\(^1\) The protocol avoided multiple iterations and manual selection of mutations by employing Rosetta enzyme design to obtain, in a single step, variants that catalyze amination of structurally demanding ketones. The presented methodology for broadening the substrate scope showed a high hit rate (68 out of 70 designs were successful) and good predictive power (Figure 1). The screening framework is computationally inexpensive (~2 CPU hours per enzyme variant using the HP workstation Z4 with an Intel Xeon W-2135 processor) and can easily be parallelized using multiple cores for high-throughput screening. Another attractive feature of the protocol is that combinations of mutations can be found using the Rosetta search algorithm, without the need to preselect them by rational inspection; only the search space needs to be defined. Other useful methodologies for predicting the catalytic potential of \(\omega\)-TA variants (using a different intermediate) are computationally more expensive (e.g., ~60 CPU hours per enzyme variant in the methodology described by Voss et al.\(^17\)) or make use of coarser categorizations such as active vs inactive in the work of Sirin et al.\(^18\) and high- vs low-reactivity grouping used by Han et al.\(^21\) A study in which the activity toward 2-acetylbiphenyl ketone was engineered in \(\psi\)TA followed an approach in which mutations were added in a stepwise manner, without predicting activity for a large number of variants as a ranking tool.\(^20\)

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of the substrates. The selected substrates are highly hydrophobic, which means modest direct participation from water molecules, which are typically deleted before docking. The substrates are also rigid, which, on one hand, further reduced the necessary search space and, on the other hand, made finding better variants more challenging. For substrates with high flexibility and high hydrogen-bonding potential, attention should be paid to both correct modeling of implicit or explicit water and including a good diversity of rotamers in the search space. However, this remains a serious challenge, especially due to the complexity of describing the behavior of water molecules.36

The design algorithm repeatedly suggested the replacement of Trp58 and Phe86 of PjTA-R6 to smaller hydrophobic residues (Table 1). In view of the sequence similarities of VjTA (PDB 4E3Q, 41% identity with PjTA-R6), CvTA (PDB 4A6T, 40% identity with PjTA-R6), and ωTA from Ochrobactrum anthropi (OuTA, PDB 5GHF, 64% identity with PjTA-R6), it is not surprising that the equivalent positions also strongly influenced activity when tested in these enzymes. The best variant of OuTA possessed mutation W58A and displayed a 105-fold higher activity in the synthesis of 1b than its parent, that is, 90 mU/mg.21 Although the curves in Figure 5 indicate that amine synthesis rates drop during conversion, the initial rates of amine formation we summarize in Table 2 are higher or comparable to this value.

In OuTA variants designed for 2a and 3a, mutation W58L relieved steric hindrance in the large binding pocket and gave a 760-fold increase in activity with 2a (3 mU/mg) and 210-fold increase in activity with 3a (0.7 mU/mg) compared to the parent.15 Replacing Trp58 of OuTA also promoted activity with other aliphatic and aromatic ketones, as seen with mutants W58L and W58A.23,24 In VjTA, the corresponding Trp57 was mutated to Phe or Cys in several designs for the synthesis of hydrophobic bulky amines.15,20 In the redesign of VjTA for asymmetric synthesis of (1S)-1-(1,1′-biphenyl-2-yl)ethanamine, the best variant-containing mutation W57F exhibited 1716-fold higher activity in comparison to the wild-type VjTA.20 Mutation W57G was beneficial for the activity of VjTA toward different aliphatic amines,20 while W57F gave improved activity with different aliphatic aldehydes and (R)-ethyl 5-methyl 3-oxocyclooctane.18,59,60 The corresponding position in CvTA is Trp60, and mutation W60C improved the activity in the deamination of (S)-1-phenylethylamine and the amination of a series of ketones.36 ωTA mutants in which the position equivalent to Phe86 of PjTA is mutated have been reported as well. Phe86 flanks the small binding pocket and was mutated to Leu to produce several variants of CvTA (F88L) and VjTA (F85L) that have higher activity with 1a and gave higher yield of 1b.17,18 Replacement of Phe86 to the smaller Leu was enough to create room for the propyl group of 1b in the small binding pocket. When VjTA was engineered to produce 1b, the best variant-containing mutation F85L gave 53% yield. Similarly, the best variant of CvTA possessed the corresponding mutation F88L and exhibited 99% conversion in the synthesis of 1b. Other small residues that improved activity in the synthesis of bulky amines when replacing Phe86 are Ala11,18,60 and Val.15

The computational approach presented in this work avoids multiple design iterations and instead uses the Rosetta energy function in a single dock-and-design step. Rosetta found solutions that overlap with rational design approaches, pointing at the conserved Trp and Phe residues in the large and small binding pocket, respectively, as hotspots for selectivity engineering of the well-studied fold type I ω-TAs. The results show that it is possible to rapidly design transaminase variants with improved activity in the production of bulky amines. The structures predicted by Rosetta design calculations are in agreement with experimental 3D structures as determined by protein crystallography and explain the observed changes in activity. We advocate that active site redesign by covalent docking of external aldimines with simultaneous sequence optimization offers a cheap and straightforward design methodology that can be applied to broaden the substrate scope of transaminases.

**ASSOCIATED CONTENT**

 Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.1c02053.

Substrate and intermediate structures, experimental details, chromatograms, docking structures, description of libraries, results of MD simulations, and crystallographic data (PDF)

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Q.M. and C.R.-P. contributed equally. Q.M. constructed mutants and performed the biocatalytic experiments. C.R.-P. performed the computational work. N.C., H.J.R., and A-M.W.H.T. solved the crystal structures. M.E.H. contributed to biocatalytic experiments. All authors discussed the results. Q.M., C.R.-P., and N.C. wrote the manuscript. D.B.J., S.J.M., H.J.W., and S.T. revised the manuscript. D.B.J. supervised the project.

Notes
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ABBREVIATIONS
ω-TA, ω-transaminase; PLP, pyridoxal 5-phosphate; PMP, pyridoxamine 5-phosphate; P/TA-R6, stabilized ω-TA from Pseudomonas jessenii; IPA, isopropylamine; CvTA, ω-TA from Chromobacterium violaceum; VfTA, ω-TA from Vibrio fluvialis; OsTA, ω-TA from Ochrobactrum anthropi; dRFU/dT, change in fluorescence versus temperature

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