



A Promiscuous De Novo Retro-Aldolase Catalyzes Asymmetric Michael Additions via Schiff Base Intermediates**

Xavier Garrabou, Tobias Beck, and Donald Hilvert*

Abstract: Recent advances in computational design have enabled the development of primitive enzymes for a range of mechanistically distinct reactions. Here we show that the rudimentary active sites of these catalysts can give rise to useful chemical promiscuity. Specifically, RA95.5-8, designed and evolved as a retro-aldolase, also promotes asymmetric Michael additions of carbanions to unsaturated ketones with high rates and selectivities. The reactions proceed by amine catalysis, as indicated by mutagenesis and X-ray data. The inherent flexibility and tunability of this catalyst should make it a versatile platform for further optimization and/or mechanistic diversification by directed evolution.

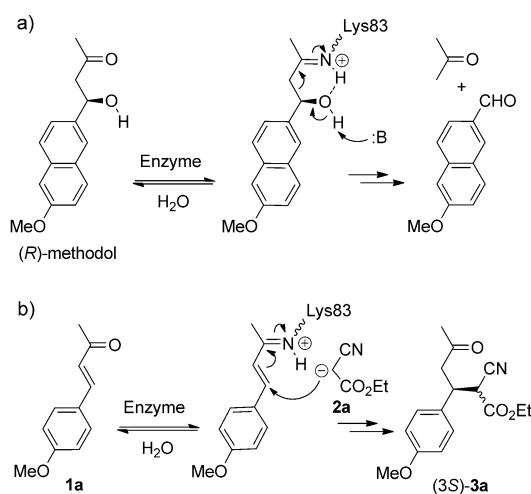
Creating enzymes with novel activities and specificities is challenging.^[1] Computational methods offer a potentially general means of designing such molecules. Powerful algorithms have been used to produce primitive enzymes for a range of mechanistically diverse reactions, including non-biological transformations.^[2] Although the starting activities are typically modest, directed evolution can afford significant improvements in rate and selectivity.^[3] As such catalysts rely on rudimentary sets of functional groups, they might also be good starting points for divergent evolution.

Retro-aldolases that exploit amine catalysis are the mechanistically most complex enzymes designed to date.^[2b,3a,4] They utilize a reactive active-site lysine to promote a multistep reaction sequence involving several enzyme-bound Schiff base intermediates. Following laboratory evolution, activities approaching those of natural class I aldolases have been attained.^[3b] Nevertheless, when compared to natural aldolases,^[5] the designed binding pockets of these enzymes are relatively unsophisticated, consisting primarily of a reactive amine in an apolar binding pocket.^[3b,6] Given this

simplicity, evolutionary optimization has sometimes resulted in extensive active-site remodeling.^[3b] We hypothesized that it might also confer promiscuous activities that could be enhanced and diversified by protein engineering.^[7] In this respect, such catalysts might complement simple amine-based organocatalysts,^[8] channeling reactive intermediates along specific trajectories, facilitating greater stereocontrol, and offering higher reaction rates. Our initial efforts focused on C–C bond formation through Michael reactions.

RA95.5-8, which efficiently cleaves (*R*)-4-hydroxy-4-(6-methoxy-naphthalen-2-yl)butan-2-one [(*R*)-methodol, see Scheme 1 a], has a snug pocket for the methyl group adjacent to the carbonyl moiety and a large hydrophobic binding site to accommodate the aromatic substituent.^[3b] A set of unsaturated methyl ketones was therefore prepared and tested as potential Michael acceptor substrates (Supporting Information, Scheme S1).^[9] The ketones were expected to react with Lys83, the catalytic amine, to form a covalently bound iminium intermediate, which could be attacked by appropriate nucleophiles. Michael additions of **1a–1g** with a range of donor molecules (**2a–2e**) were performed in the presence of RA95.5-8 and monitored by HPLC (see the Supporting Information for details). Although most combinations afforded only small amounts of the new products, the reaction of **1a** and ethyl 2-cyanoacetate (**2a**) was particularly rapid and high-yielding (Scheme 1 b). The resulting Michael adduct (**3a**) was isolated and fully characterized.

Analysis of **3a** revealed high enantiocontrol at the site of addition (3*S*/3*R* = 98:2), whereas the central carbon atom of



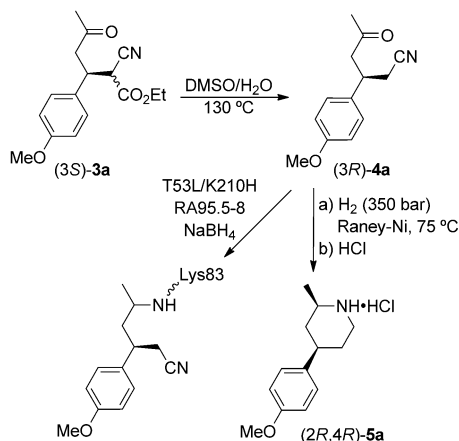
Scheme 1. Retro-aldol and Michael reactions catalyzed by the computationally designed and evolved RA95 enzymes.

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the donor moiety freely epimerized in water. The absolute configuration of the enzymatically controlled chiral center was elucidated by subjecting the Michael adduct to a Krapcho decarboxylation and a subsequent reduction as previously described,^[10] followed by X-ray analysis of the hydrochloride salt of the chiral cyclic amine (2*R*,4*R*)-**5a** (see Scheme 2 and the Supporting Information).



Scheme 2. Preparation of the cyclic amine (2*R*,4*R*)-**5a** and derivatization of T53L/K210H RA95.5-8 with the decarboxylated Michael adduct (3*R*)-**4a**.

To optimize this promiscuous activity, nine positions in the active site were individually randomized, and improved variants were identified by monitoring depletion of **1a** at 350 nm in a spectrophotometric kinetic assay in multi-well plates. Two mutations, T53L and K210H, enhanced the catalytic efficiency by a factor of approximately three without significantly compromising the enantioselectivity of the reaction (3*S*/3*R* = 96:4). The steady-state kinetic parameters for the Michael addition catalyzed by T53L/K210H RA95.5-8 were obtained by globally fitting the data to a random binding mechanism. The intersecting lines in the double-reciprocal plot (Figure S3) indicate independent binding of **1a** and **2a** to the active site.^[11] Notably, the turnover number of T53L/K210H RA95.5-8 ($k_{\text{cat}} = 0.217 \pm 0.004 \text{ s}^{-1}$) is comparable to that of RA95.5-8 for the cleavage of (*R*)-methodol ($k_{\text{cat}} = 0.36 \text{ s}^{-1}$). This value corresponds to a 6300-fold rate acceleration over the spontaneous background reaction. The K_{M} value for unsaturated ketone **1a** ($322 \pm 12 \mu\text{M}$) is similar to that for (*R*)-methodol with RA95.5-8 ($230 \mu\text{M}$), suggesting an analogous binding mode, whereas the high K_{M} value for donor **2a** ($16.5 \pm 0.5 \text{ mM}$) may be attributed to the lack of an explicitly evolved binding site. The steady-state parameters obtained with a variant containing only the K210H mutation (Tables S2 and S3, Figure S4) indicate that this substitution primarily lowers the apparent K_{M} value for the Michael acceptor, whereas the T53L mutation is responsible for the observed increase in k_{cat} . Residue 53 is in close proximity to Lys83 and may favorably perturb the $\text{p}K_{\text{a}}$ value of the catalytic residue.

The improved synthetic utility of the mutant manifested itself in the enantioselective Michael addition of **2a** to

acceptors that were poorly converted by RA95.5-8, such as (*E*)-4-phenylbut-3-en-2-one (**1b**) and (*E*)-4-(6-methoxynaphthalen-2-yl)but-3-en-2-one (**1c**; see the Supporting Information). These results underscore the potential of the retro-aldolase for evolutionary optimization. Multiple rounds of random mutagenesis and screening might be used to create a higher-affinity binding site for the donor, improve the reactivity of poorer nucleophiles, or achieve the stereocontrolled installation of additional chiral centers.

Direct insights into the catalytic mechanism were obtained by crystallization of T53L/K210H RA95.5-8 in complex with a stable derivative of the Michael product. The enzyme was modified with (3*R*)-**4a**, and the resulting Schiff base adduct was reduced with NaBH₄ (Scheme 2). The structure of the complex, which was determined to 1.20 Å resolution, shows the ligand covalently bound to Lys83 with its aromatic substituent occupying the apolar binding site designed to bind the naphthyl ring of methodol (Figure 1A). These observations support the hypothesis that Lys83 functions as an amine catalyst in the enzymatic transformation, increasing the electrophilicity of the acceptor through Schiff base formation. Replacement of Lys83 with methionine led to a 100-fold decrease in activity (see Table S2), providing further corroboration for this mechanism. Although the precise disposition of the reactants at the active site is unknown, the X-ray structure suggests that the covalently bound Schiff base adopts an extended conformation that exposes the *Re* face of the olefin to attack at C4 by the carbanion donor, which likely binds in a small, solvent-accessible pocket formed between the tips of the L1 and L6 loops.

Comparison of T53L/K210H RA95.5-8 with structurally characterized intermediates in the evolution of RA95.5-8 highlights a number of salient differences in structural motifs near the active site (Figure 1B), which underscore the pronounced flexibility of the scaffold. Compared to the previously determined RA95 structures,^[3b] loops L1 (residues 51–64) and L6 (residues 180–190), which displayed a range of conformations and high thermal displacement parameters in the other variants, are now extensively disordered. Most of L1, which contains the T53L mutation, was not resolved, whereas two conformations could be modeled for a few residues in L6. Although the residues in direct contact with the ligand were not observed, the occupancy of one of the L6 conformations correlates with that of the covalently bound ligand, suggesting that the Michael adduct induces (partial) structure in this floppy segment. Loop L7 (residues 211–215), adjacent to the K210H mutation but far from the bound ligand, also adopts a completely different backbone conformation than in other RA95 structures. Furthermore, the α -helix formed by residues 233–239, consistently structured in previous variants, is distorted in T53L/K210H RA95.5-8. The marked conformational flexibility of this scaffold is likely to be a significant factor in fostering mechanistic promiscuity.^[12]

The ability of the artificial RA95.5-8 retro-aldolase to activate an unsaturated ketone as an iminium ion enables efficient catalysis of asymmetric Michael additions. This activation mode is unique among the few reported examples

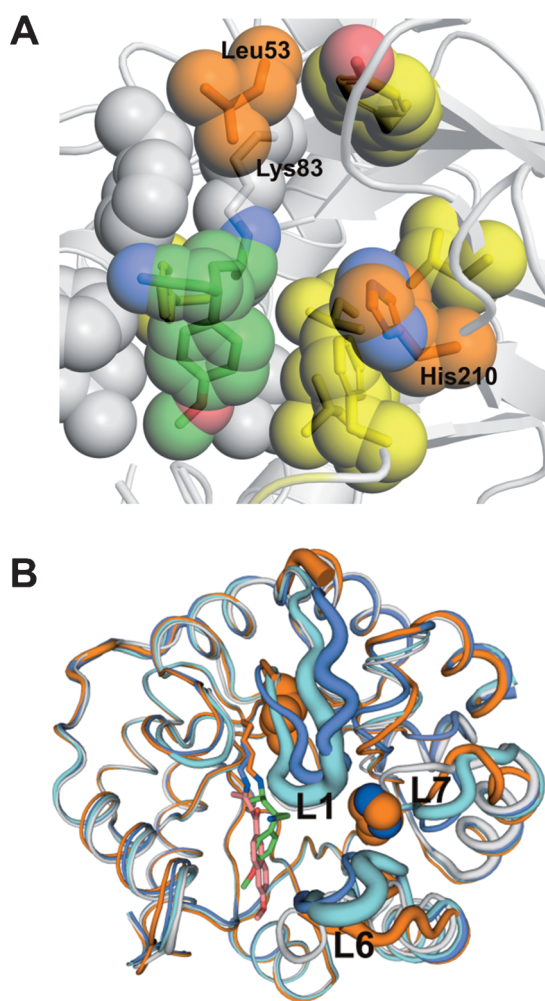


Figure 1. A) Crystal structure of T53L/K210H RA95.5-8 and the Michael adduct derivative (4R)-4a (in green) after chemical reduction of the adduct covalently bound to Lys83. Positions subjected to randomization are highlighted in yellow, and the mutations introduced in this work are depicted in orange. B) Alignment of T53L/K210H RA95.5-8 (orange, the mutated positions 53 and 210 are highlighted as spheres) derivatized with (4R)-4a (green) and the previously solved structures of RA95.0 (pale gray), RA95.5 (cyan), and RA95.5-5 (blue) derivatized with a diketone analogue of methodol (pink). For each structure, the distribution of the thermal displacement parameters within the model is represented by the tube diameter.

of Michael reactions catalyzed by promiscuous enzymes, which either increase the reactivity of the Michael acceptor through hydrogen-bonding interactions with the oxanion hole of lipases^[13] or activate aldehyde-based Michael donors by enamine formation.^[14] Although high catalytic rates^[13a] and enantioselectivities have been reported for these systems,^[14b,15] the combination of catalytic proficiency ($[k_{\text{cat}}/(K_{\text{M,acceptor}} \times K_{\text{M,donor}})]/k_{\text{uncat}} = 1.2 \times 10^9 \text{ M}^{-1}$) and stereochemical control achieved by T53L/K210H RA95.5-8 is remarkable for an artificial enzyme.

In summary, our results indicate that computationally designed retro-aldolases are a potentially valuable source of promiscuous catalytic activity. As the chemistry of iminium ions and enamines, formed through attack of an amine on

a carbonyl group, is broad in scope but mechanistically simple, these catalysts could conceivably accelerate many of the carbon–carbon bond-forming reactions promoted by organo-catalysts. Moreover, such promiscuity could be quite general. Although computational methods are not yet able to create the precisely tailored active sites of highly evolved natural enzymes, the construction of binding pockets containing reactive acids, bases, and nucleophiles appears to be relatively straightforward.^[2b,c,16] It is likely that the promiscuous activities of these proteins can be amplified and refined by directed evolution to generate diversified families of artificial enzymes with broadened substrate and reaction scope.

Keywords: asymmetric catalysis · biocatalysis · directed evolution · enzyme design · enzyme promiscuity

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