Engineering Carboxylic Acid Reductase for Bio-based Chemical Synthesis

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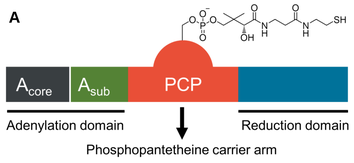
Background

Enzymes are large biological catalysts that enable chemical transformations for life. Enzymes are also essential in a wide range of industrial applications.[1]

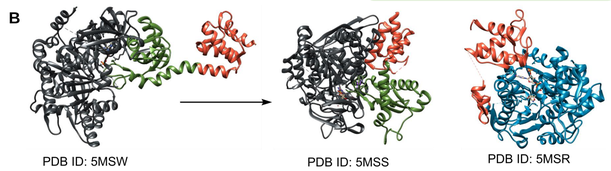
Problem: Kinetic performance of an enzyme may limit its industrial use.

How to overcome this limitation? We can improve the catalytic properties and substrate scope of the enzyme via protein engineering.[2]

Carboxylic Acid Reductase (CAR)s (EC 1.2.1.30) are large multi-domain enzymes that can catalyze the ATP- and NADPH-dependent reduction of carboxylic acids into aldehydes. These enzymes consist of three domains: the adenylation domain (A-domain), the phosphopantetheine carrier protein domain (PCP-domain), and the reduction domain (R-domain).[1, 3]



CARs are **valuable tools for biosynthesis** due to the[1]:
- Broad substrate scope
- Relaxed reaction conditions
- Domain modularity



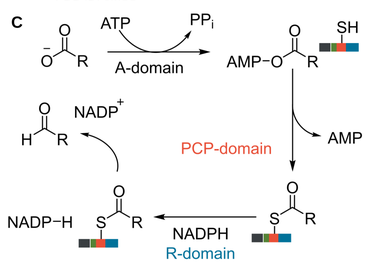


Figure 1. Structure and function of CARs. (A) Schematic representation of the structure of CAR. (B) Crystal structure of *Segniliparus rugosus* CAR (SrCAR). Color code: A-domain A_{core}, grey; A-domain A_{sub}, green; PCP-domain, red; R-domain, blue. (C) Overview of CAR catalytic mechanism.

Methods

We optimized a novel growth-coupled selection method for the engineering of NADPH-dependent enzymes, i.e., CARs.[2]

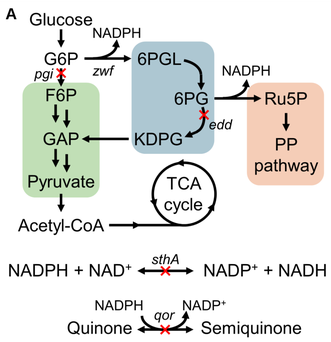
Host strain engineering: : The genes *pgi* (G6P isomerase) and *sthA* (soluble transhydrogenase) were deleted from *E. coli* genome to construct R-Δ2. Further deletion of *edd* (phosphogluconate dehydratase) and *qor* (quinone oxidoreductase) led to host R-Δ4.

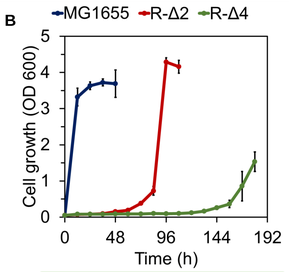
Library construction: Single-site (SSM) and multi-site saturation mutagenesis (MSM) libraries of *Mycobacterium avium* CAR (MavCAR) were constructed with the NNK codon (N = A, C, T, or G, K = T or G).

Growth-coupled selection of CAR mutants: The SSM libraries were transformed into *E. coli* R-Δ2 containing the phosphopantetheinyl transferase-expressing (SFP) plasmid. MSM library was transformed into *E. coli* R-Δ4 containing the SFP plasmid. Cells were washed and plated on M9 glucose agar plates with 10 mM adipate. Colony formation and growth were monitored.

Activity assay: Kinetic assay was performed by monitoring the oxidation of NADPH at 340 nm at 25 °C.

Growth-Coupled NADPH Recycling Strategy





R-Δ4 displayed **severe growth retardation** with an **extended lag phase.**

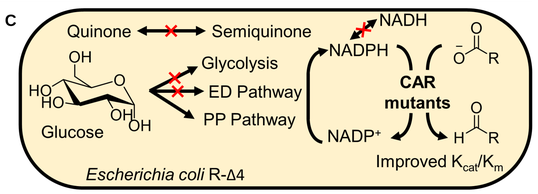
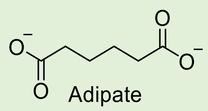
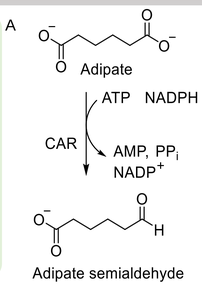


Figure 2. (A) Schematic of the overaccumulation of NADPH by the deletion of *pgi*, *sthA*, *edd* and *qor* (green: glycolysis; blue: Entner-Doudoroff (ED) pathway; red: pentose phosphate (PP) pathway). (B) Growth characterization of *E. coli* strains in minimal media with glucose. (C) Schematic illustration of the growth-coupled NADPH recycling approach used for selecting CAR enzymes with enhanced kinetics.

Engineering of CAR toward Adipate



The global demand for **bio-based polymers** is on the rise, fueled by[4]:
- Reduction of carbon footprints
- Transition to renewable feedstocks
- Increasing consumer focus on sustainability



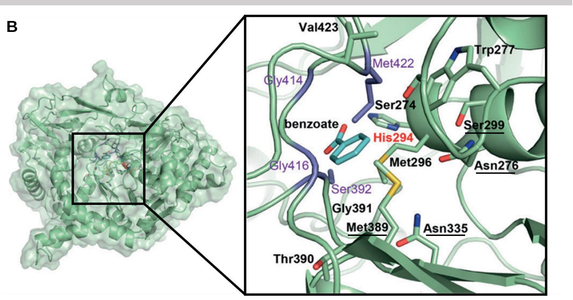


Table 1. Kinetic characterization of MavCAR variants

Variant	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m (mM^{-1} min^{-1})
wild-type	62.0 ± 3.9	59.5 ± 1.6	1.0 ± 0.1
Single-site saturation			
N335R	2.6 ± 0.1	44.9 ± 0.6	17.0 ± 1.0
S299K	4.4 ± 0.2	30.1 ± 0.3	6.9 ± 0.3
M389K	5.4 ± 0.3	32.3 ± 0.4	5.9 ± 0.4
N276P	11.1 ± 0.5	47.3 ± 0.5	4.2 ± 0.2
N276R	13.2 ± 0.5	49.3 ± 0.6	3.7 ± 0.2
S299R	13.3 ± 0.8	45.2 ± 0.9	3.4 ± 0.2
Multi-site saturation			
NSRS	2.7 ± 0.08	120.6 ± 1.0	44.5 ± 1.4
NSRQ	2.4 ± 0.31	69.1 ± 3.1	29.5 ± 3.2
SSRS	6.5 ± 0.6	44.5 ± 1.8	6.8 ± 0.6
LSRS	3.9 ± 0.1	24.8 ± 0.3	6.4 ± 0.3

Improvement compared to WT

N335R: 17-fold
S299K: 7-fold
M389K: 6-fold
N276P: 4-fold
N276R: 4-fold
S299R: 3-fold
NSRS: 45-fold
NSRQ: 30-fold
SSRS: 7-fold
LSRS: 6-fold

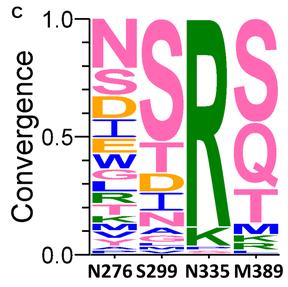
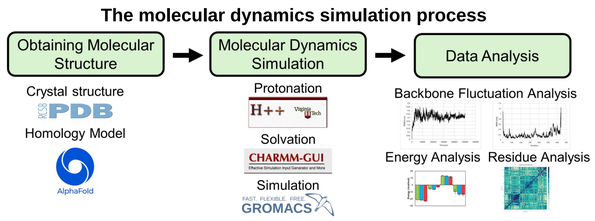
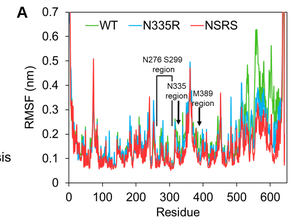
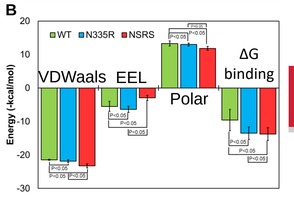


Figure 3. (A) Overview of the synthesis of adipate semialdehyde by CAR. (B) Homology model of MavCAR adenylation domain docked with benzoate and AMP. Residues chosen for engineering are in black, residues used in the MSM are underlined, and catalytic His 294 is in red. (C) Sequence logo highlighting the convergence of amino acids within MavCAR mutants.

Impact of Mutations on Function







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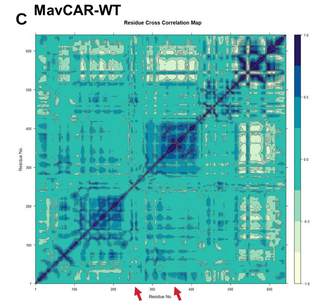

Figure 4. (A) RMSF analysis of MavCAR mutants. (B) Binding free energies with MMPBSA method. (C) Dynamic Cross-Correlation Matrix (DCCM) of MavCAR Adenylation domain bound with AMP and Adipate.

The charge alterations in **N335R** modified **electrostatic interactions** within the binding pocket, leading to an **increase in anti-correlated** regions and a **rise in the ΔEEL** energy value.

The **NSRS** mutant exhibited **significant decorrelation** between Res276 and other residues, disrupting the dynamic network but **enhancing van der Waals** interactions (lower ΔVDWaals energy). This **reduction in desolvation penalties** and **favorable binding free energy** collectively **improved catalytic efficiency**, resulting in an increased turnover number.

Conclusion and Future Work

- The growth-coupled NADPH recycling strategy allows the identification of MavCAR variants with enhanced activity toward less favorable substrates.
- Selection using MSM libraries and the improved strain allowed the identification of mutants with even better activity than those from the SSM library.
- The DCCM and binding energy results revealed how the N335R and NSRS mutations remodel both protein dynamics and energetics.
- Future work will focus further engineering CARs toward less explored substrate.

Acknowledgements

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