



Engineering Carboxylate Reductases for Activity on Dicarboxylates

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Background

Current fossil fuel reserves are rapidly depleting and many industrial chemicals are derived from petroleum. An alternative is to use chemical synthetic pathways in biological organisms, or biosynthetic pathways, to produce those molecules. Carboxylic acid reductase (CAR) enzymes can serve as catalysts in a biosynthetic pathway and convert carboxylic acids into their corresponding aldehydes.¹ Using traditional chemical synthetic methods to produce aldehydes from carboxylic acids is challenging. Meanwhile, aldehydes are useful intermediates as they can be readily converted to other useful functional groups, such as alcohols and amines.

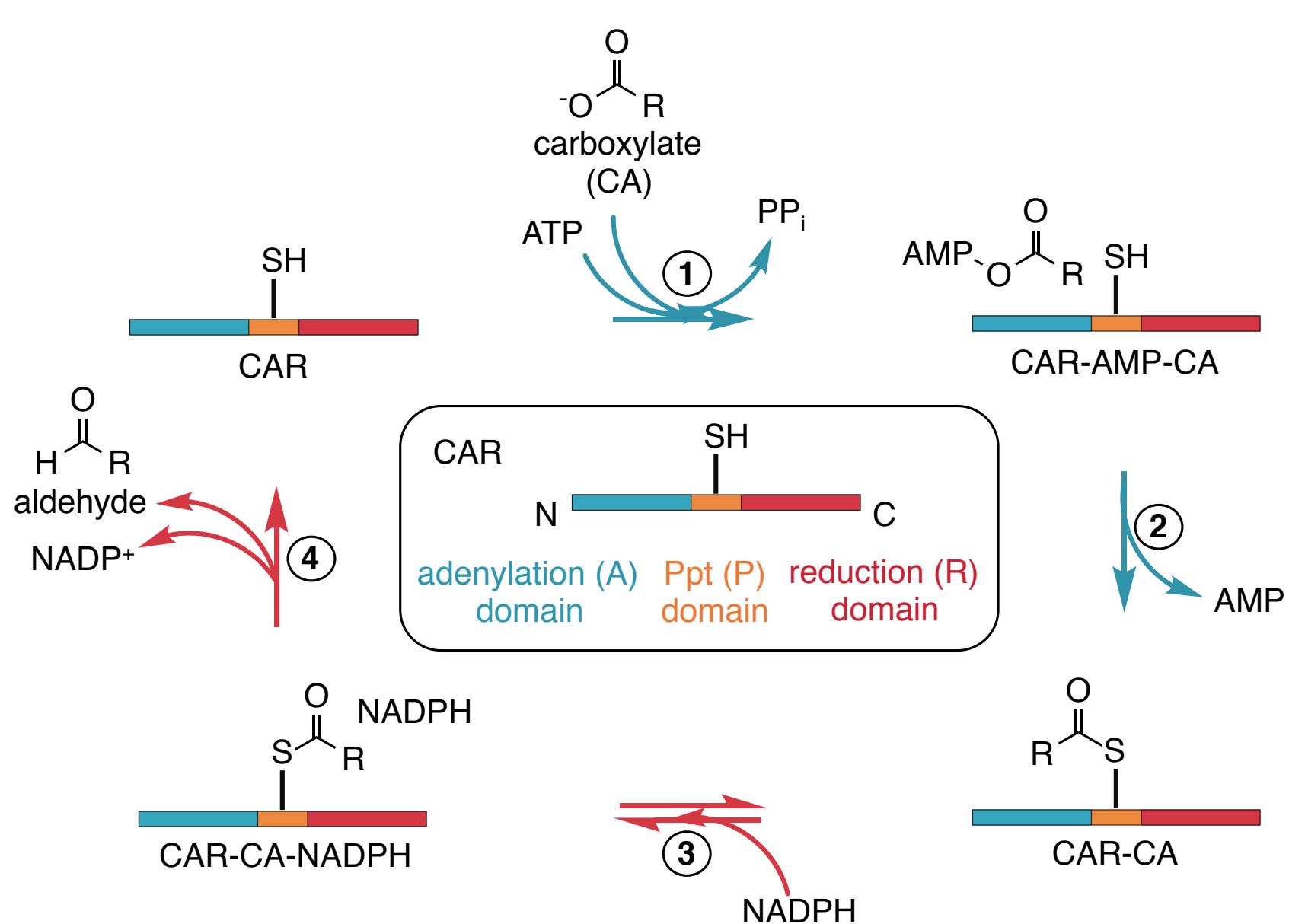


Figure 1. Catalytic mechanism and domain structure of CAR. 1. adenylation, 2. formation of the thioester intermediate, 3 and 4 reduction of the thioester intermediate.

CAR provides a less challenging pathway and makes use of the readily available carboxylic acids found in biological organisms. The CAR enzyme uses ATP and NADPH to reduce a carboxylic acid to an aldehyde. This work focuses on engineering CAR enzymes for improved activities on glutaric and adipic acid, which can be produced by engineered microbes, and were chosen to benefit the practical application of CAR.

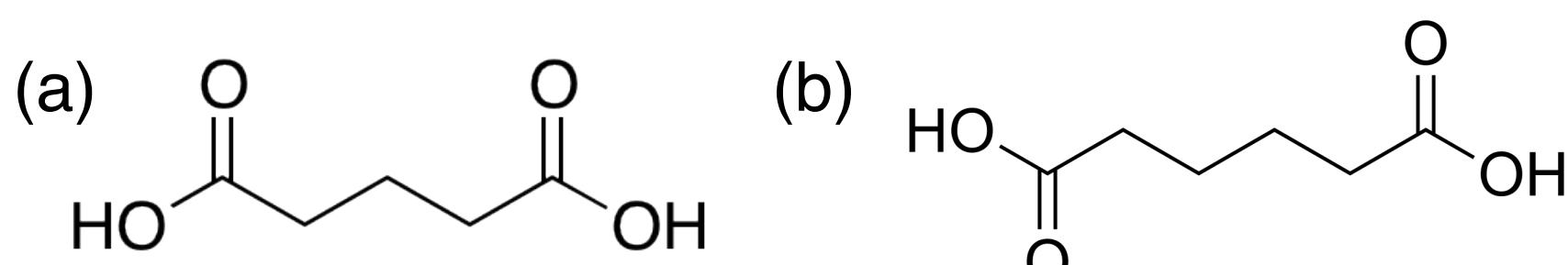


Figure 2. (a) Glutaric acid and (b) adipic acid.

Methods

Cloning & Transformation

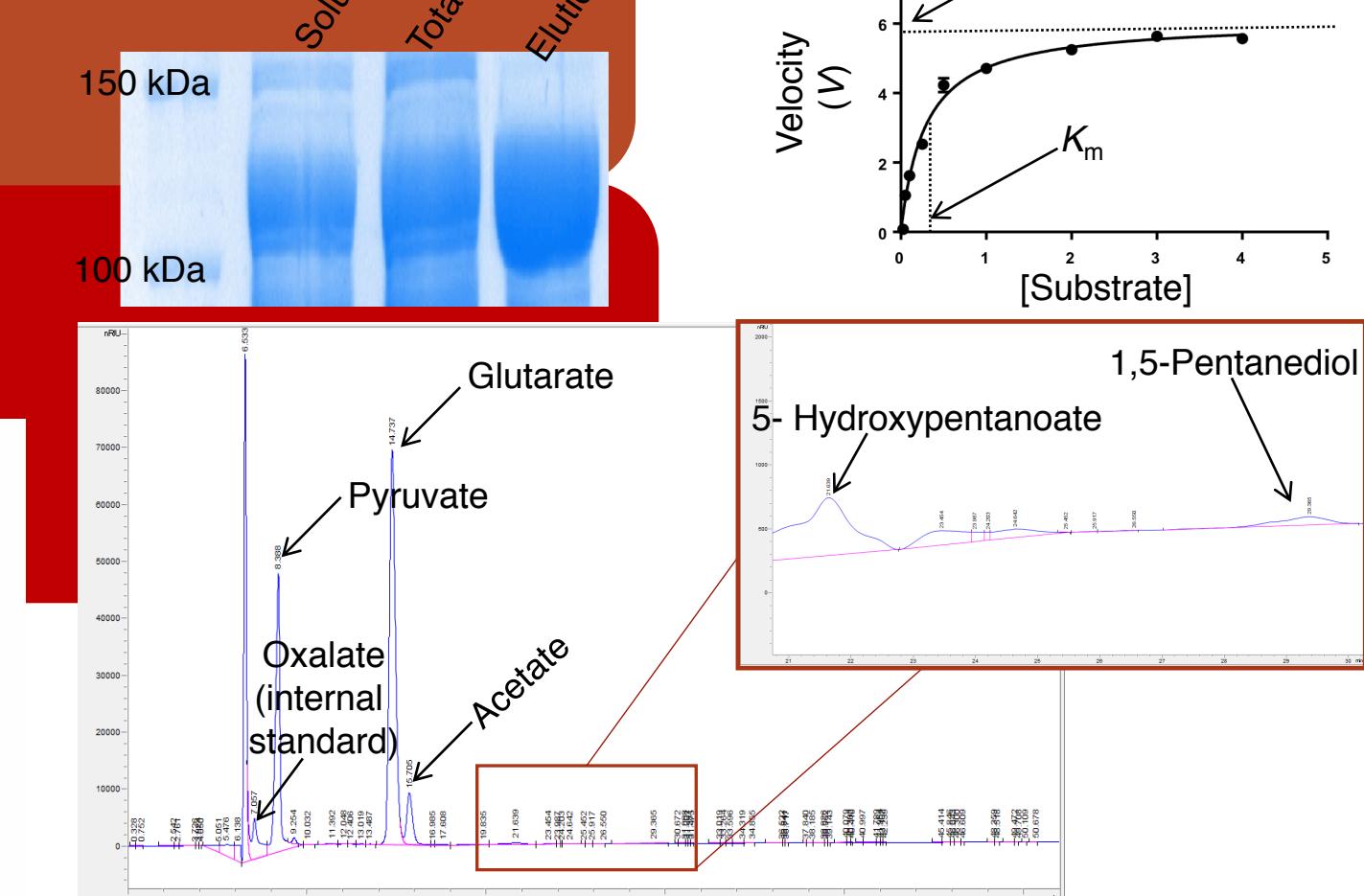
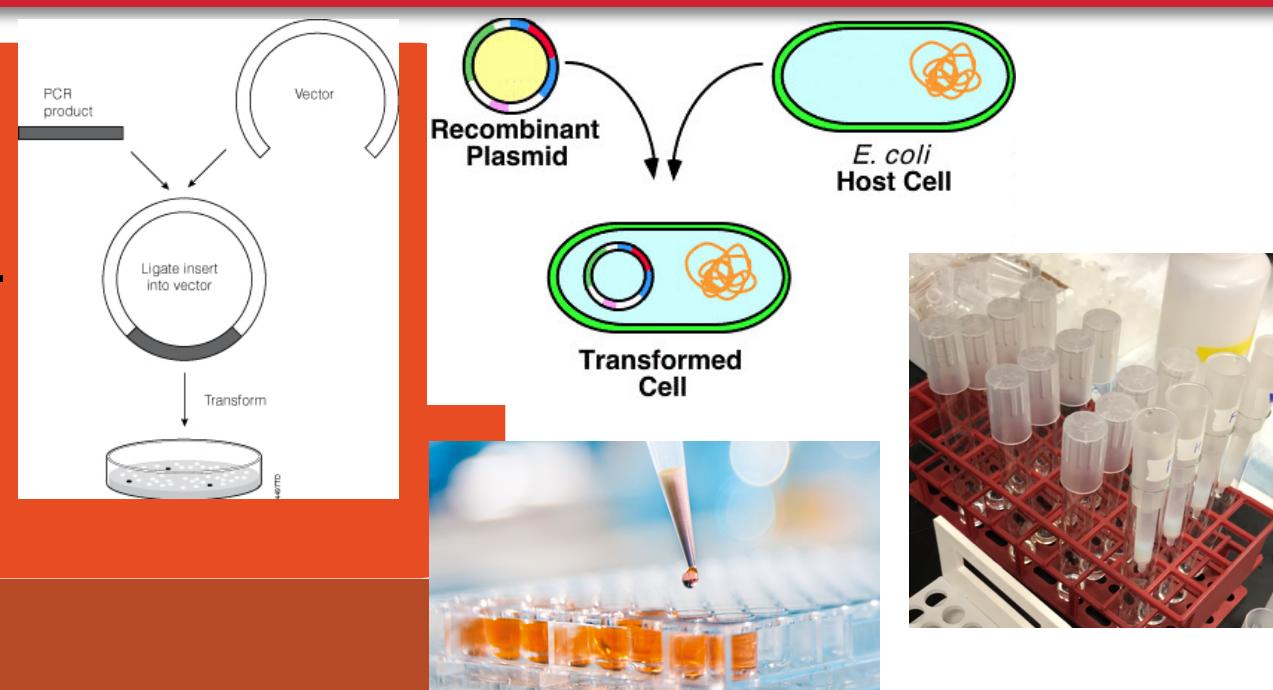
- Plasmid DNA molecules with genes encoding *M. avium* CAR mutant enzymes were constructed using sequence- and ligation-independent cloning (SLIC).²
- Cloned vector transformed into *E. coli* host using heat shock method

In vitro Characterization

- IPTG induction to grow up *E. coli* culture and overexpress mutation
- Nickel chromatography purification
- Sonication to lyse open cells

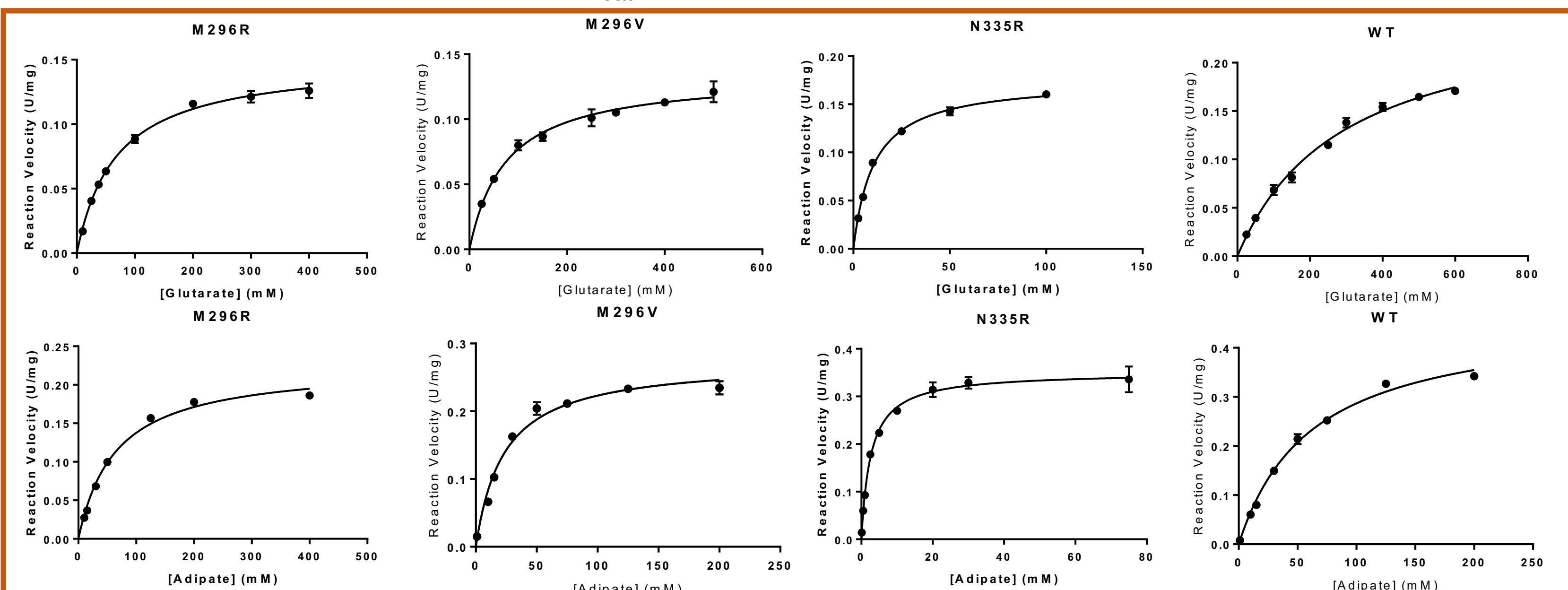
In vivo Characterization

- E. coli* cultured in M9 minimal media for 72hr
- Analyzed using HPLC or NMR



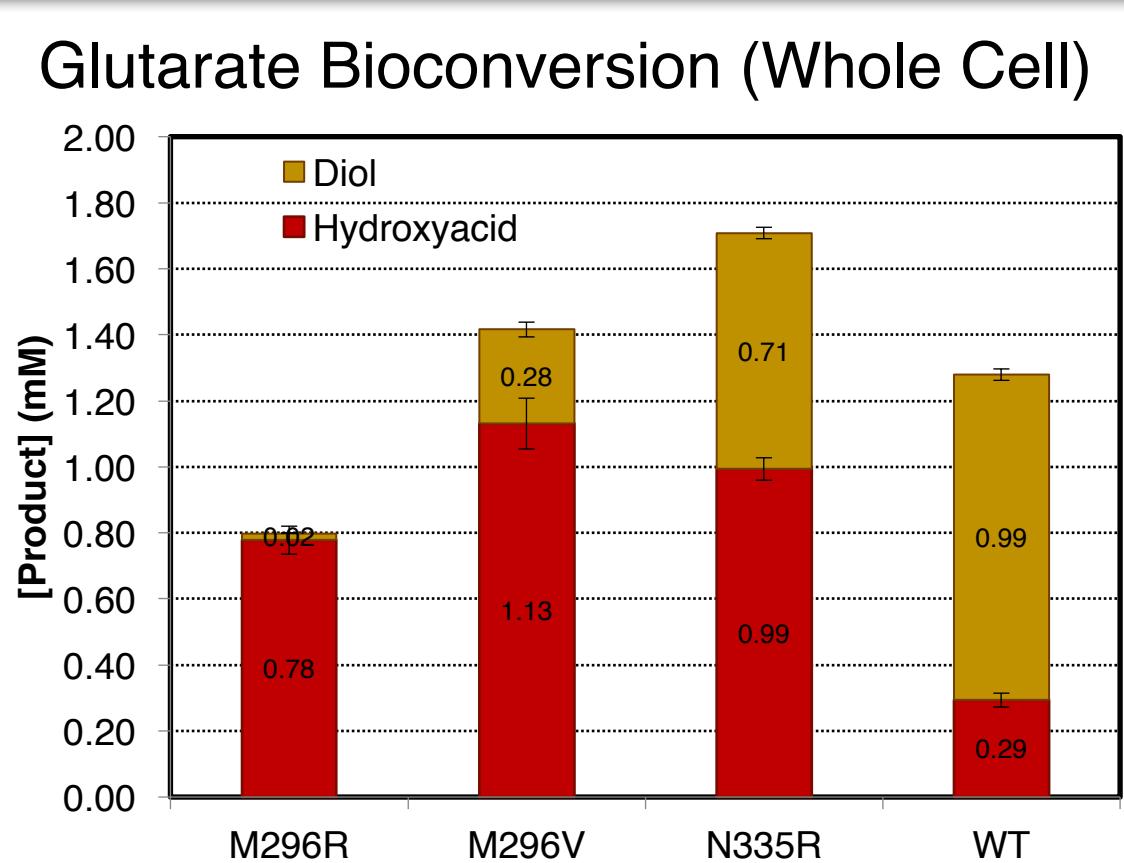
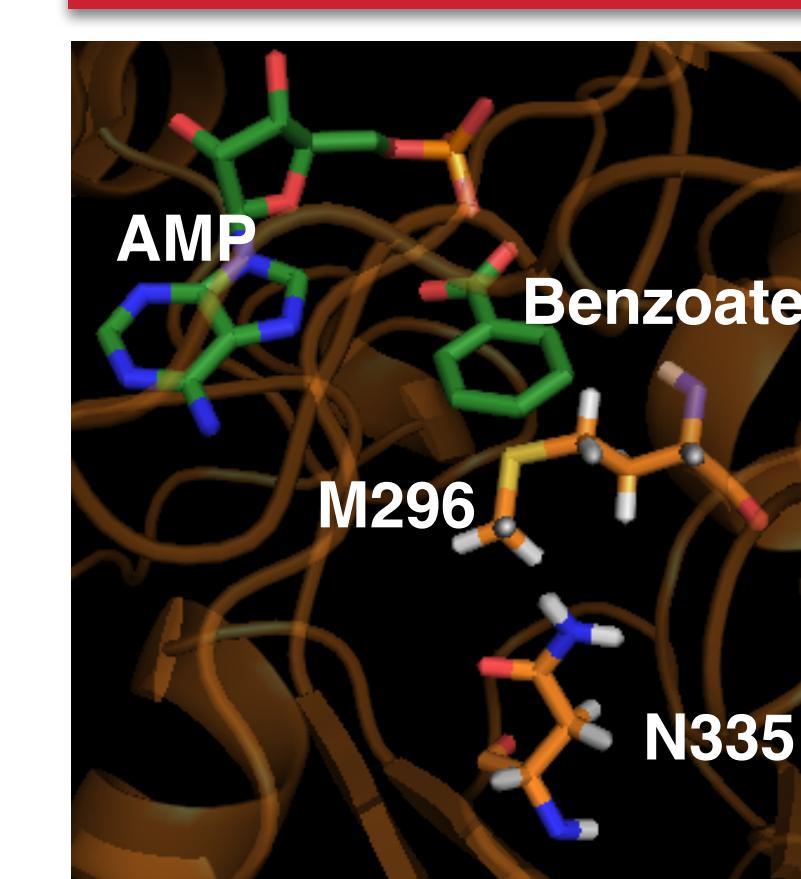
Results

Catalytic efficiency, or k_{cat}/K_m ratio, is used to measure enzyme performance. The Michaelis-Menten model constant is K_m , and k_{cat} is the substrate turnover rate.³

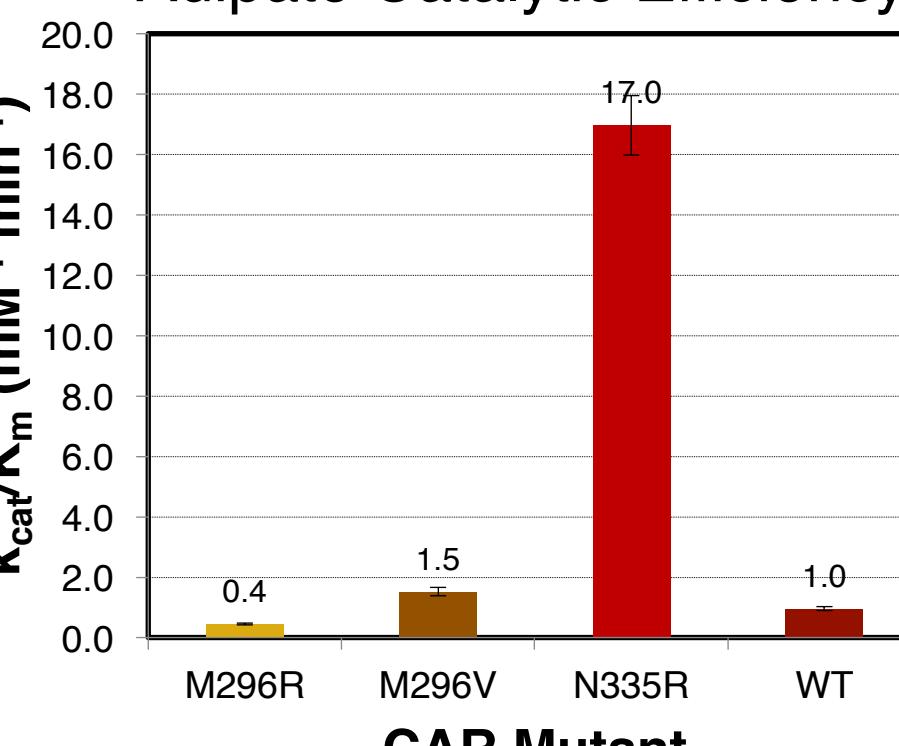
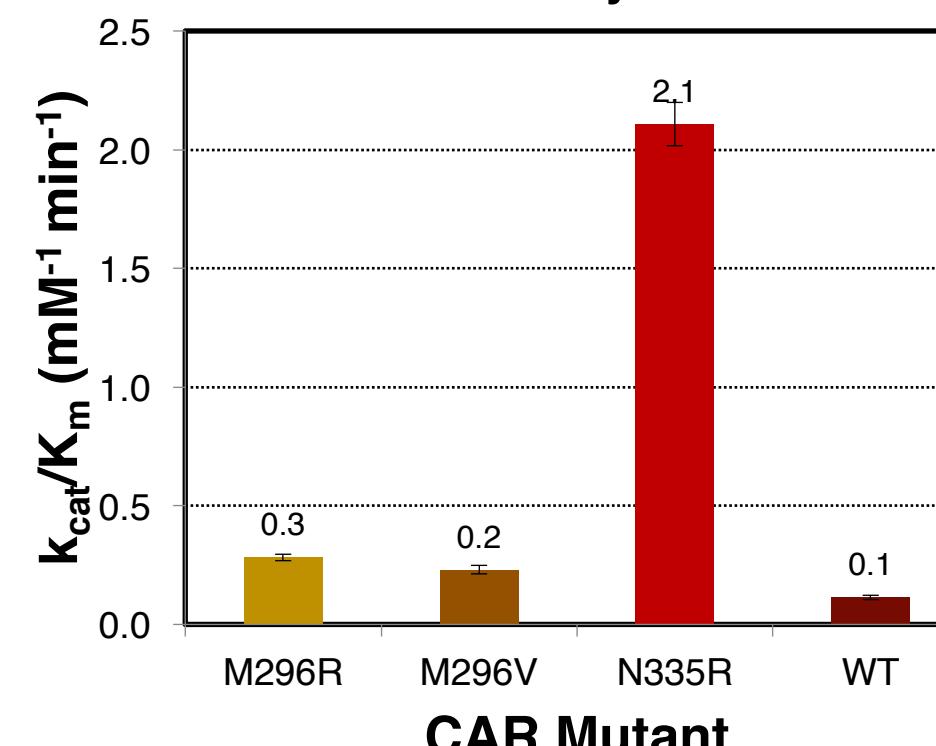


CAR Residue	Adipate		Glutarate	
	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})
M296R	64.5 ± 4.1	29.0 ± 0.6	67.9 ± 3.1	19.2 ± 0.3
M296V	23.1 ± 2.0	35.2 ± 0.9	74.3 ± 5.6	17.2 ± 0.4
N335R	2.6 ± 0.1	44.9 ± 0.6	10.6 ± 0.4	22.4 ± 0.3
WT	62.0 ± 3.8	59.5 ± 1.6	291.8 ± 21.0	33.2 ± 1.1

Results



Glutarate Catalytic Efficiency



Conclusion

- N335R mutant showed the most improved catalytic efficiency as compared to WT, and therefore most potential to be used in the practical application of CAR.
- K_m of the multidomain CAR enzyme can be changed by only engineering the adenylation domain, which supports the hypothesis that the adenylation domain plays a key role in substrate recognition

Acknowledgements

This research is funded by NSF grant CBET 1805528 and the UNL Summer Research Program.

References

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