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Engineering and characterization of hybrid carboxylic acid reductases

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ABSTRACT

Carboxylic acid reductases (CARs) are valuable biocatalysts due to their ability to reduce a broad range of carboxylate substrates into the corresponding aldehyde products. CARs are multi-domain enzymes with separate catalytic domains for the adenylation and the subsequent reduction of substrates. Inter-domain dynamics are crucial for the catalytic activities of CARs. In this work, hybrid enzymes that contain domains from four bacterial CARs and one fungal CAR were constructed based on domain boundaries that were defined using a combination of bioinformatics and structural analysis. Hybrid CARs were characterized in both steady-state and transient kinetics studies using aromatic and straight-chain (C_3-C_5) carboxylate substrates. Kinetic data support that the inter-domain interactions play an important role in the function of both wild-type and hybrid CARs and further lead to the hypothesis that reduction is the rate-determining step in CAR catalysis. Our results provide both fundamental insights into CAR catalysis and rationale for hybrid CAR engineering.

1. Introduction

Carboxylic acid reductases (CARs) are multi-domain enzymes that catalyze an NADPH-dependent reduction of carboxylates into corresponding aldehydes through an *in situ* ATP-consuming activation mechanism. (He et al., 2004; Venkitasubramanian et al., 2007a,b) Based on protein sequence analysis, CARs are categorized into four subgroups, including one bacterial (type I) and three fungal (type II-IV) groups (Stolterfoht et al., 2017; Winkler, 2018). CARs in general display broad tolerance to substrate structures. Activities towards aromatic, long-chain aliphatic, short-chain amino, short-chain hydroxyl, cyclic carboxylates and dicarboxylates were reported. (Winkler, 2018; Akhtar et al., 2013; Schwendenwein et al., 2016, Finnigan et al., 2017; Khusnutdinova et al., 2017; Kramer et al., 2018; Qu et al., 2018) Due to the diverse reactivity of their aldehyde products, which are often difficult to obtain through chemical synthesis, CARs are proven to be versatile biocatalysts in both *in vitro* enzymatic and whole-cell applications. (Winkler, 2018; Kramer et al., 2018; Qu et al., 2018; Kunjapur et al., 2014; France et al., 2016; Moura et al., 2016; Horvat et al., 2019; Ramsden et al., 2019; Strohmeier et al., 2019) The catalytic cycle of CARs is executed through the highly orchestrated activities of three domains, including an N-terminal adenylation domain (A), a C-terminal reduction domain (R), and an internal phosphopantetheine attachment site/domain (P), which is post-translationally modified and serves the key function of shuttling the catalytic intermediates from the A domain to the R domain (Fig. 1). (Venkitasubramanian et al., 2007a; Gahloth et al., 2017; Qu et al., 2019) Structures of CAR domains from several bacterial species were solved in a recent study. (Gahloth et al., 2017) Furthermore, individual CAR domains were shown to be catalytically functional. (Gahloth et al., 2017; Wood et al., 2017) This provides an opportunity to systematically construct and characterize hybrid CARs consisting of domains from different parent enzymes. Building hybrid enzymes from domains/subunits of different origins is a successful strategy in both mechanistic studies and the engineering of multi-domain/multi-subunit enzymes, such as polyketide synthases (PKSs) and nonribosomal peptide synthases (NRPSs). (Tsuji et al., 2001; Broadhurst et al., 2003; Hahn and Stachelhaus, 2004; Weissman and Mueller, 2008) In this study, we constructed 16 CARs using one single domain and one di-domain of either bacterial or fungal origin. These hybrid CARs were systematically characterized in both steady-state kinetics and transient kinetics studies. Mutagenesis experiments were performed to provide further insights into the role of domain interactions in the function of hybrid CARs.

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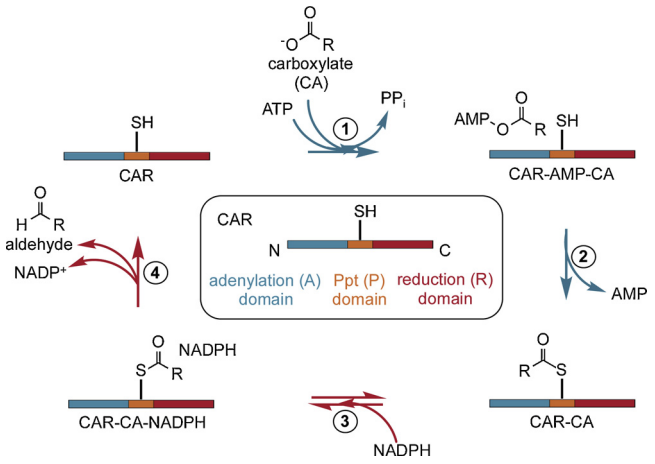

Fig. 1. Domain structure and catalytic mechanism of CARs. 1. adenylation, 2. formation of the thioester intermediate, 3. and 4. reduction of the thioester intermediate.

2. Results and discussion

2.1. Construction of hybrid CARs

CARs from four bacterial strains, including *Mycobacterium avium* (Mav), *Mycobacterium marinum* (Mm), *Kutzneria albida* (Ka), and *Nocardia iowensis* (Ni), and one fungal strain, *Neurospora crassa* (Nc), were chosen as parents. (Schwendenwein et al., 2016; Kramer et al., 2018) A total of four sets of hybrid CARs were constructed by pairing either a single domain, i.e. A or R, or a di-domain, i.e. AP or PR, from the MavCAR with CAR domains of another origin (Fig. 2A). The MavCAR domain was included in all designs due to the superior kinetic properties of the enzyme. (Kramer et al., 2018) Domains in each enzyme were identified based on protein sequence alignment using the Clustal Omega program. (Sievers et al., 2011) The exact domain boundaries of the four Type I bacterial CARs were chosen by taking account of reported structural data of CAR domains (Fig. 2B). (Gahloth et al., 2017) Although the Type IV fungal NcCAR and Type I bacterial CARs have low overall sequence homology, the phosphopantetheinylated serine residues of fungal and bacterial CARs were aligned correctly (data not shown). Based on this result, we defined the boundary between the A and P domains of NcCAR at residue 547, the P and R domains at residue 648. Plasmids encoding hybrid CARs were constructed using the sequence- and ligation-independent cloning method from PCR products of CAR domains (Li and Elledge, 2007). C-terminal His6 affinity tags were included for protein purification.

Table 1
Kinetic properties of wild-type and hybrid bacterial CARs on benzoate.

entry	CAR	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
1	*M. avium*	1.20 ± 0.12	214.9 ± 6.8	180 ± 20
2	*M. marinum*	0.23 ± 0.04	33.9 ± 1.0	148 ± 27
3	*K. albida*	0.24 ± 0.06	171.5 ± 7.9	723 ± 187
4	*N. iowensis*	1.11 ± 0.07	135.2 ± 2.6	122 ± 8
5	Mav(A)-Mm (PR)	0.45 ± 0.04	72.2 ± 1.5	161 ± 16
6	Mav(AP)-Mm (R)	0.50 ± 0.05	65.8 ± 1.4	132 ± 14
7	Mm(A)-Mav (PR)	0.34 ± 0.07	162.2 ± 10.0	474 ± 102
8	Mm(AP)-Mav (R)	0.07 ± 0.02	16.7 ± 0.9	235 ± 63
9	Mav(A)-Ka(PR)	1.01 ± 0.12	213.5 ± 6.6	211 ± 26
10	Mav(AP)-Ka(R)	0.45 ± 0.06	86.6 ± 2.8	193 ± 27
11	Ka(A)-Mav(PR)	0.39 ± 0.04	267.3 ± 7.6	692 ± 83
12	Ka(AP)-Mav(R)	0.46 ± 0.09	87.3 ± 4.4	190 ± 38
13	Mav(A)-Ni(PR)	0.54 ± 0.05	40.4 ± 1.0	75 ± 8
14	Mav(AP)-Ni(R)	0.27 ± 0.01	63.2 ± 7.1	234 ± 27
15	Ni(A)-Mav(PR)	0.76 ± 0.06	190.3 ± 3.8	250 ± 20
16	Ni(AP)-Mav(R)	0.77 ± 0.06	117.4 ± 2.3	153 ± 12

2.2. Kinetics characterization of hybrid CARs

Wild-type and hybrid CARs were co-expressed with the phosphopantetheinyl transferase (Sfp) of *N. iowensis* in an *E. coli* host. Enzymes were purified using nickel affinity chromatography. Concentrations of purified enzymes were determined by Bradford assay. The steady-state kinetic properties of purified CARs were examined on the benzoate substrate by following previously reported method. (Kramer et al., 2018) All bacterial hybrid CARs are functional (Table 1). When comparing kinetic properties among the four hybrids generated from the same two parent enzymes (entry 5–8, 9–12, or 13–16 in Table 1), poor correlations of K_m values between parents and hybrids were observed. On the other hand, hybrids containing a natural PR di-domain showed faster turnover rates than ones containing the natural AP di-domain from the same CAR. In addition, bacterial hybrids consisting of the MavCAR PR di-domain paired with another A domain (entry 7, 11, 15 in Table 1) had the highest k_{cat} and the highest catalytic efficiencies out

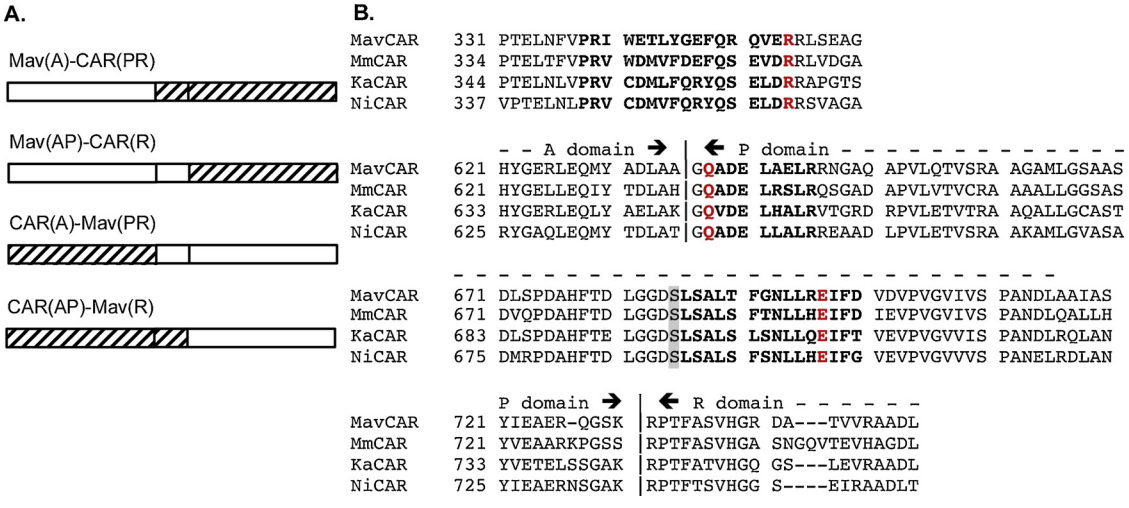

Fig. 2. Design principles of hybrid CARs. A. Graphic representation and nomenclature of hybrid CARs. Open squares represent domains of MavCAR. Shaded squares represent domains of other CARs, including MmCAR, KaCAR, NiCAR, and NcCAR. B. Domain boundaries in Type I bacterial CARs. Sequences of potentially interacting helices are in boldface. The phosphopantetheinylated serine residue is highlighted.

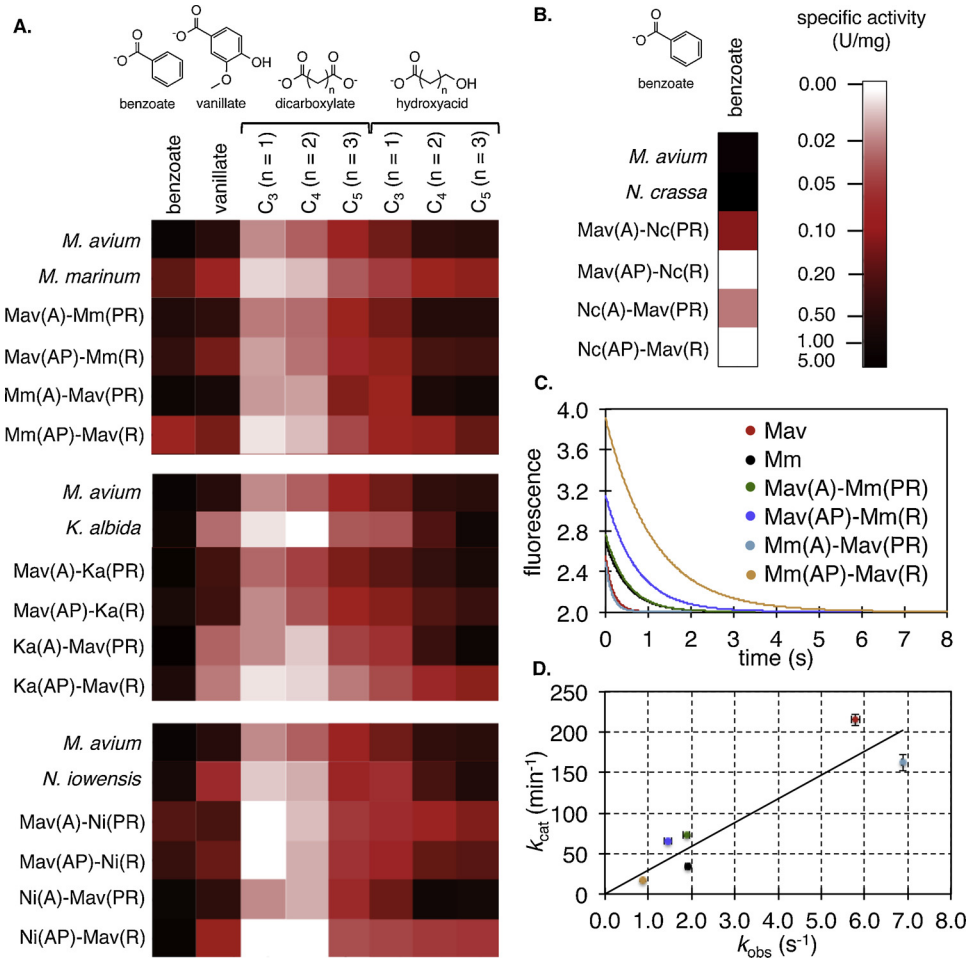

Fig. 3. Substrate scope of wild-type and hybrid CARs. A. Specific activities of parent and hybrid bacterial CARs. B. Specific activities of parent and hybrid bacterial-fungal CARs. C. Traces of stopped-flow measurements. D. Comparison between steady-state (k_{cat}) and single-turnover (k_{obs}) kinetics. Dicarboxylates: C_3, malonate; C_4, succinate; C_5, glutarate. Hydroxyacids: C_3, 3-hydroxypropionate; C_4, 4-hydroxybutyrate; C_5, 5-hydroxypentanoate. Heat maps show the average specific activities in U/mg from three replicates. Heat maps were generated in CIMMiner. The scale bar is for both graphs A and B. Graphs C and D share the same legend. Substrate concentrations are 5 mM for aromatic carboxylates, 200 mM for C_4-C_5 hydroxyacids and 500 mM for other carboxylates.

of all four hybrids of the same parents. The substrate scope of bacterial hybrid CARs was also examined on benzoate, vanillate, C_3-C_5 hydroxyacids and dicarboxylates (Fig. 3A). The hybrid CARs showed similar substrate specificity to that of the parent enzymes, which are more active on substrates of longer carbon chain length and prefer a hydroxyacid over a dicarboxylate of the same carbon chain length. (Kramer et al., 2018) Furthermore, the same trend in activity ranking was observed among the four hybrids derived from the same two parents as in the kinetics analysis in Table 1. Hybrid enzymes with a natural PR di-domain showed higher specific activities, while enzymes containing the MavCAR PR di-domain in general showed the highest specific activities. The only exception is the Mav(AP)-Ni(R) hybrid, which displayed better kinetic properties and specific activities than that of Mav(A)-Ni(PR). Only low overall activities were observed for the four hybrid enzymes derived from bacterial MavCAR and fungal NcCAR. Therefore, no detailed kinetics analysis was carried out. Nevertheless, a similar trend was observed where hybrids containing the natural PR di-domain showed higher specific activities on benzoate substrate (Fig. 3B).

2.3. Domain dynamics of hybrid CARs

To understand the reason that hybrid CARs with natural PR di-domain in general showed better kinetic properties, we first conducted single-turnover reduction kinetics analysis of MavCAR, MmCAR, and their four hybrids using benzoate as the substrate. Purified enzymes were first incubated with benzoate and ATP for 20 min to form the thioester intermediate (Fig. 1). Following a desalting step, reduction of the catalytic intermediate by NADPH was analyzed using a stopped-flow apparatus by monitoring the fluorescence decrease (Fig. 3C). The observed rate constants (100 μM enzymes) were calculated by fitting fluorescence traces to exponential decay functions in Kinetic Studio software. A linear correlation between the k_{obs} in single-turnover analysis and the k_{cat} in steady-state experiments was observed (Fig. 3D). Since above single-turnover experiment measures the overall rate of steps 3 and 4 in Fig. 1, which potentially include the cooperative movement of the P and R domains to engage in a catalytically active conformation, the results indicate that the reduction half-reaction is critical in determining the overall rates of CAR-catalyzed reactions. More detailed studies are required to test this hypothesis. Since hybrid CARs with natural PR di-domains maintained interactions that are critical to the dynamics between the P and R domains of a wild-type enzyme, they therefore showed better kinetic properties than the ones with natural AP di-domains. This observation is consistent with early studies of PKSs and NRPSs, which revealed that protein-protein interactions at the interface of domains or subunits play a critical role in determining enzymatic activities. (Tsuji et al., 2001; Weissman and Mueller, 2008; Dehling et al., 2016)

Engineering the interface interaction is proven to be an effective strategy in the construction of functional chimeric enzymes for combinatorial biosynthesis. (Dehling et al., 2016; Walsh, 2002; Hahn and Stachelhaus, 2006) Since hybrid CARs with natural PR di-domain showed better kinetic properties, we next studied how the interactions between the A and P domains were affected in this type of hybrid CARs. To identify key residues that contribute to the potential interactions, we first examined the domain interface of the AP di-domain of the wild-type *Segniliparus rugosus* CAR (SrCAR). Anchoring of the P domain to the A domain appears to be enabled by interactions between three helices. One resides in the middle of the A domain (a.a. 360–376; helix 1), while the other two reside at the N-terminus (a.a. 653–662; helix 2) and in the middle (a.a. 703–717; helix 3) of the P domain, respectively

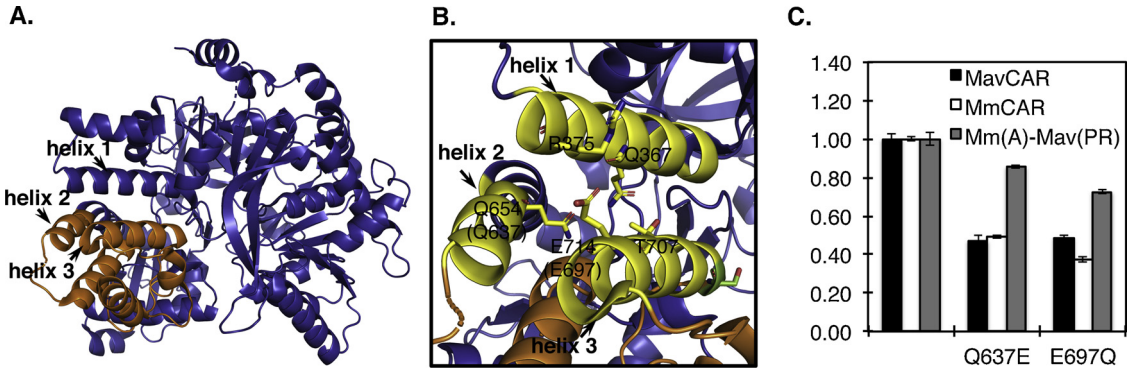

Fig. 4. Interface between A and P domains. A. Crystal structure of AP di-domain of *S. rugosus* CAR at the thiolation state (PDB, 5MSS). A domain, blue; P domain, orange. B. Close-up of the three helices (yellow) at the A and P domain interface of SrCAR in graph A. Possible interacting residues are labeled. Equivalent residues in MavCAR are shown in parenthesis. C. Relative specific activities of CAR mutants. Data are represented as the average of six replicates with standard deviation (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Fig. 4A, 4B). Sequence alignment showed the corresponding residues in the four bacterial CARs that were used as the parents in this study (Fig. 2B). A Q637E and an E697Q mutant of MavCAR were constructed to disrupt potential interactions at the interface. Enzyme assays showed that the Q637E mutant retained 47.1 ± 2.6%, while the E697Q mutant retained 48.8% ± 1.0% of activities on benzoate substrate (Fig. 4C). Residues Q637 (Q654 in SrCAR), E697 (E714 in SrCAR), and R354 (R375 in SrCAR), which potentially engage in electrostatic interaction with E697 (Fig. 4B), are conserved among the four bacterial CARs (Fig. 2B). Indeed, when each of the two mutations was introduced into MmCAR, a 49.1 ± 0.8% and a 37.6 ± 1.5% retention of activities were observed, respectively (Fig. 4C). Above data in Fig. 4C indicate that key interactions at the interface of A and P domains are necessary for the function of wild-type CARs. In addition, due to high sequence similarity, such interactions are likely to be preserved in bacterial hybrid CARs, which enable their function. It also provides one explanation to the observed low activities of bacterial-fungal hybrids, where key interactions at domain interface were likely disrupted due to low sequence similarity. When mutations Q637E and E697Q were introduced into hybrid CAR, Mm(A)-Mav(PR), reduction in activities was also observed, albeit to a less extent (Fig. 4C). The data supports the hypothesis that the effective A and P domain interface was maintained in the bacterial hybrid CARs. Meanwhile, lower levels of activity decrease in comparison to those of MavCAR and MmCAR mutants indicate that new domain interface might be formed in the hybrid CAR, which further emphasized the complexity of domain dynamics in CAR catalysis.

3. Conclusions

This work reports the construction of hybrid CARs containing domains with different origins based on domain boundaries that were determined in previous CAR crystallization efforts. (Gahloth et al., 2017) Characterization of hybrid CARs provided both practical guidelines for hybrid CAR engineering and insights into the fundamental mechanism of CAR catalysis. Better kinetics observed in hybrid CARs that preserve a natural PR di-domain suggest that retaining the cognate P and R domains benefits the construction of highly functional hybrid CARs. Reduced activities were observed in mutagenesis experiments that focused at the A and P domain interface of wild-type and hybrid bacterial CARs. The results emphasize the importance of maintaining proper domain interaction(s) in the catalytic process of CARs, while loss of such interaction may contribute to low or no activity of bacterial-fungal hybrid CARs. Meanwhile, due to the lack of structural data of fungal CARs, non-optimal definition of domain boundaries can also impair functions of hybrid CARs with fungal origin. Furthermore, steady-state and single-turnover kinetics analysis of wild-type and hybrid CARs lead to the hypothesis that the reduction step, which potentially also requires conformational shift between the P and R domains, is the determinant factor of the overall turnover rate in CAR catalysis.

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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