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Carboxylic acid reductase enzymes (CARs)

Margit Winkler[1,2]

Carboxylate reductases (CARs) are emerging as valuable catalysts for the selective one-step reduction of carboxylic acids to their corresponding aldehydes. The substrate scope of CARs is exceptionally broad and offers potential for their application in diverse synthetic processes. Two major fields of application are the preparation of aldehydes as end products for the flavor and fragrance sector and the integration of CARs in cascade reactions with aldehydes as the key intermediates. The latest applications of CARs are dominated by *in vivo* cascades and chemo-enzymatic reaction sequences. The challenge to fully exploit product selectivity is discussed. Recent developments in the characterization of CARs are summarized, with a focus on aspects related to the domain architecture and protein sequences of CAR enzymes.

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Introduction

Carboxylic acid reductases (CARs) catalyze the reduction of a carboxylic acid substrate to the corresponding aldehyde (Scheme 1). EC 1.2.1.30 CARs activate the carboxylate substrate at the expense of ATP and catalyze the reduction step using NADPH as the hydride donor [1]. This activity has long been observed as a metabolic reaction of numerous fungi [2••,3•] and pioneering work on the characterization of the first CAR protein, purified from the fungus *Neurospora crassa* (*Nc*CAR), was reported in the 1970s [4]. The first amino acid sequence of a CAR was elucidated from a bacterial source (*Nocardia iowensis*, *Ni*CAR) [5••]. For years, *Ni*CAR occupied a unique role in the scientific literature until the first homologous sequence was published in 2013 [6•]. In the last two years, considerable interest in CARs is reflected by new enzymes being reported from various labs worldwide, and new concepts for the application of CARs in synthetic routes. This review focuses on the last three years of research on recombinant CARs, as only sequence information allows for the application of an enzyme in biocatalysis on scale, be it *in vitro* or *in vivo*.

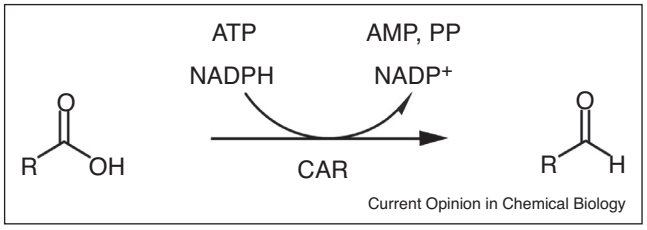
The broad substrate scope of CARs

The potential of CARs for bioorganic synthesis is underlined by the relaxed substrate tolerance of this enzyme class. Figure 1 gives a semi-quantitative comparison of CAR activities for diverse substrates, ranging from aliphatic acids to aromatic, polycyclic and heterocyclic acids. This summary includes data from recombinant CARs (type I: *Nocardia iowensis Ni*CAR #Q6RKB1.1; *Mycobacterium marinum Mm*CAR #WP_012393886.1; *Segniliparus rotundus Sr*CAR #WP_013138593.1; *Mycobacterium neoaurum Mn*CAR #WP_019810583.1; *Nocardia brasiliensis Nb*CAR #AFU02004.1; *Mycobacterium smegmatis Ms*CAR1 #WP_015306631.1 and *Ms*CAR2 #AFP42026.1; *Mycobacterium phlei Mp*CAR #WP003889896.1; *Nocardia otitidiscaviarum No*CAR #WP_029928026.1; *Tsukamurella paurometabola Tp*CAR #WP_013126039.1; type II: *Aspergillus terreus At*CAR #XP_001212808.1 *Stachybotrys bisbyi Sb*CAR #BAV19380.1; type III: *Neurospora crassa Nc*CAR #YP_955820.1; type IV: *Trametes versicolor Tv*CAR #XP_008043822.1) and data from *Ni*CAR and *Nc*CAR isolated from their natural hosts [2••,5••,6•,7–22]. Reported non-substrates are also included in Figure 1. The substrate specificities of CARs largely overlap, but there are exceptions. *Sr*CAR, for example, was the only CAR reducing a nitro-substituted benzoic acid derivative [14]. CARs were historically named aryl-aldehyde dehydrogenase ($NADP^+$), indicating selectivity for aromatic substrates, however, short to medium chain aliphatic acids are also efficiently reduced. Octanoic acid, for example, is reduced by recombinant *Ni*CAR with a k_{cat} of 233 min^{-1} [16], which is even higher than typical reports for the reduction of the aromatic standard substrate benzoic acid. In general, the substrate scope of CARs is exceptionally broad, ranging from aliphatic compounds (from C2 to C18) to (hetero)aromatic compounds and a wide range of substituents is tolerated. A clear limitation, so far, seem to be polar groups adjacent to the carboxylate moiety. This conclusion is corroborated by results of the Yakunin group [23].

Domain architecture and mechanistic features of CARs

EC 1.2.1.30 CARs consist of three domains: an adenylation domain (A-domain), a transthiolation domain (T-domain), and a reductase-domain (R-domain) (Figure 2).

Scheme 1



CARs catalyze the direct and selective two-electron reduction of organic acids to aldehydes. Four-electron reduction to alcohols has not been observed with wild-type CARs.

A conserved serine in the T-domain (Figure 2, yellow spheres) is post-translationally modified by a phosphopantetheinyl transferase [24]. The modular domain architecture with A-domains and T-domains shows similarities to non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), in which the domains are also connected by flexible linkers, and a substrate is shuttled from the *N*-terminus to the *C*-terminus. Owing to this modularity, a recent phylogenetic study suggested four distinct CAR subgroups. One group consists of bacterial (type I) and the other three groups of fungal CARs (type II–IV). The bacterial group is characterized by an extra C-terminal stretch in the A-domain (Figure 2a, salmon region) [25,26••]. Figure 2 shows models of the type I *Ni*CAR in comparison to the type III *Nc*CAR. Despite subtle differences, the overall architecture is similar. Notably, the enzymatic activity of Type II and IV CARs appears to be significantly lower in comparison to type I and III CARs (Figure 1).

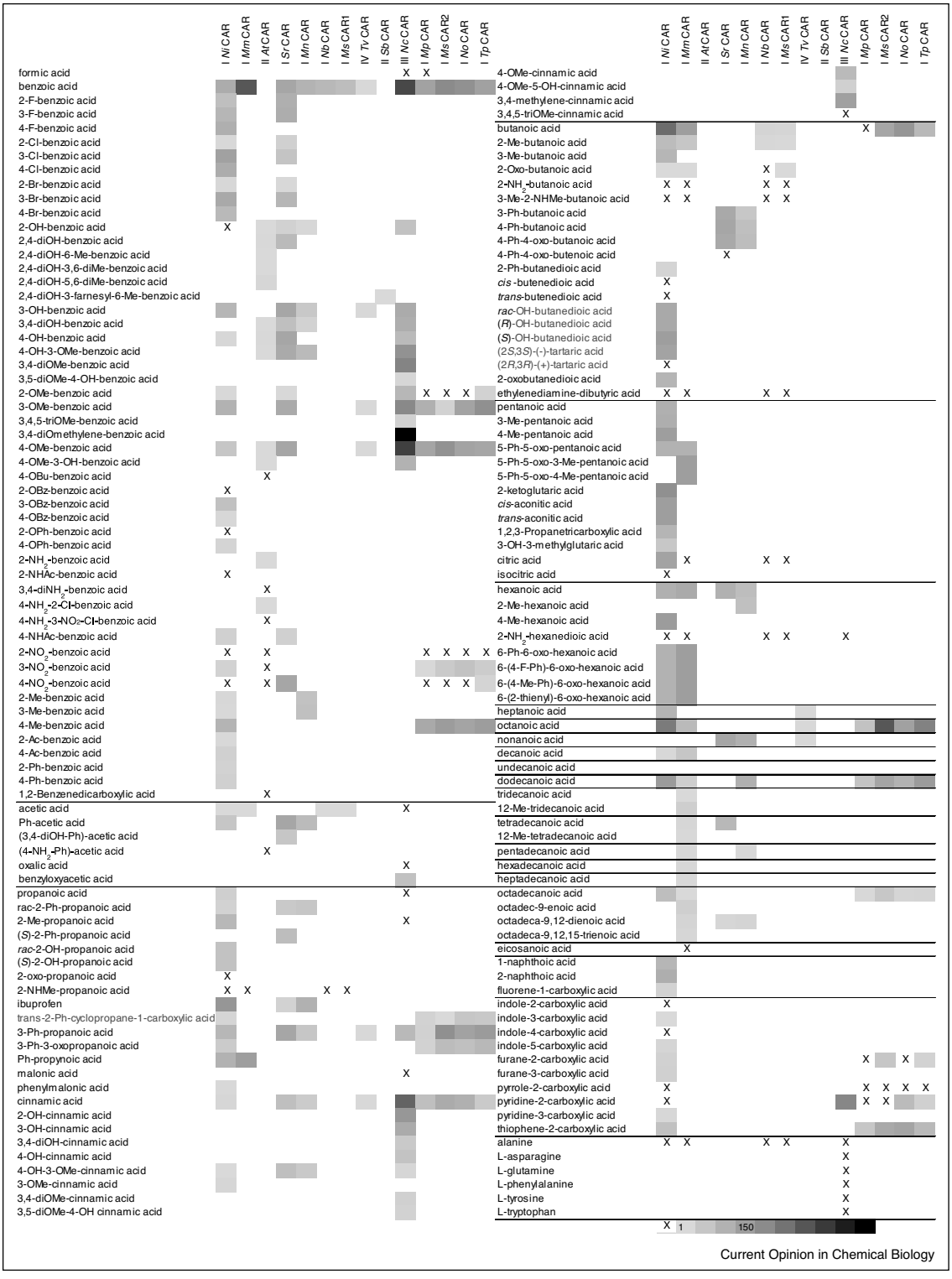
CARs start the catalytic cycle by activation of the carboxylate substrate. The precise mechanism of the reaction cascade from acid to aldehyde is not yet fully proven experimentally and represents an area for further studies. In our current understanding, the first step is the activation of the carboxylate as an AMP-ester at the expense of ATP. The second step is a transesterification of the AMP-ester by the thiol of the phosphopantetheine to a thioester, which binds the acyl moiety covalently to the CAR. Next, the T-domain turns and transfers the substrate from the A-domain towards the R-domain in a swinging arm fashion [27]. Finally, the thioester is reduced in the R-domain by NADPH [5••]. The dynamics and driving force of each single step on the molecular level needs experimental confirmation and may be rationalized by mutational and kinetic studies similarly to comprehensive NRPS domain alteration studies [28]. At present, first mutational studies are becoming available with the aim to identify key residues. The reductase domain of NRPS proteins was proposed to consist of the catalytic triad T, Y and K in similarity to a tyrosine-dependent short chain dehydrogenase family [29]. The tyrosine and lysine were confirmed to be essential in *At*CAR [30•] and *Nc*CAR [25] (Y863 and K867 as well as Y844 and K848, respectively), however, threonines are not conserved in CAR reductase domains. In the A-domain of *Nc*CAR, the exchange of H237 and E433 for alanine abolished activity, indicating a catalytic role of these residues. Structural information for full length CARs is not yet available, but a NRPS crystal structure was solved in 2016 [31]. These new insights provide a sound basis to understand CARs in more detail on the molecular level and are expected to inspire CAR engineering. Until very recently, mutational studies were guided by molecular models. A Phyre2 [32] model of type I CARs shown in Figure 2a is based on five templates for the A-domain (pdb codes 5ja2, 5t3d, 4zxi, 5es8 and 2vsq; all are NRPS ligase modules) and one for the R-domain (pdb 4dqv; a reductase domain of a NRPS from *Mycobacterium tuberculosis*). These templates form also the basis for the *Nc*CAR model, but an additional sequence (pdb 4r0m) was used as a template for the A-domain. It needs to be stressed, however, that due to low similarities of these templates and CARs, particularly in the A-domain, the quality of these models is poor. Only in July 2017, first crystal structures of individual CAR domains with and without T-domain and a full length model of a new CAR from *Segniliparus rugosus* were published [26••].

A sequence alignment of all experimentally confirmed CARs allowed to retrieve a characteristic CAR signature consisting of four regions with conserved residues in the A-domain, the region around the phosphopantetheinylated serine in the T-domain and three regions with conserved residues in the R-domain (Figure 2, green and red residues) [25]. This information is valuable to guide the identification of new CAR sequences. Owing to the modular domain architecture, domain-swapping or subdomain-swapping may be a viable engineering approach for CARs, alternatively to classical protein engineering methods [33,34].

Synthetic applications of CARs

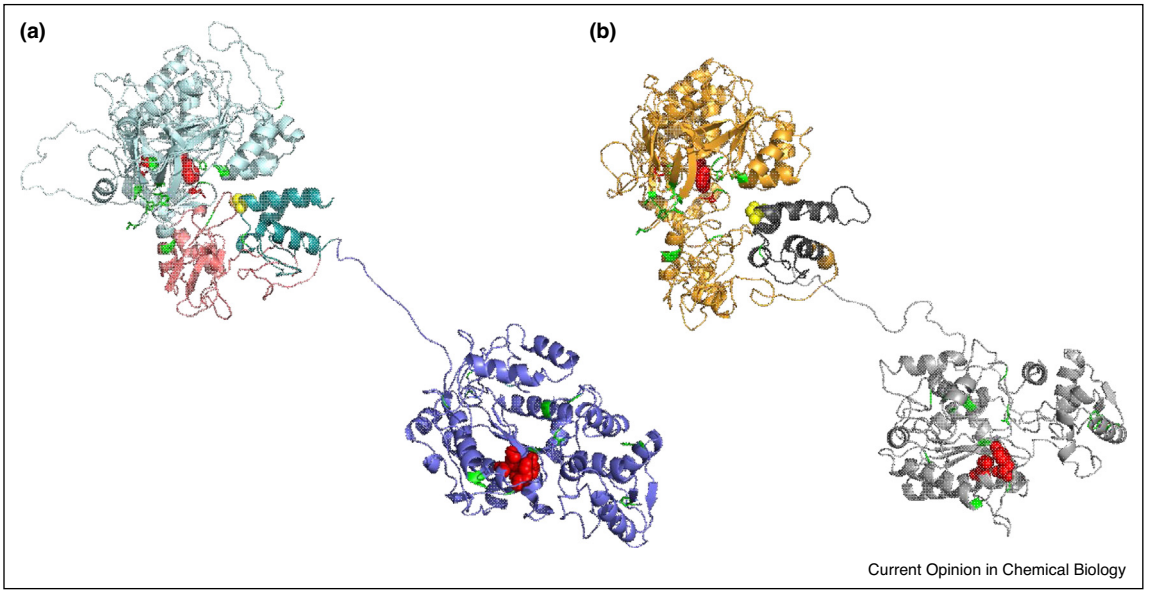
The most extensively studied application of CARs is the preparation of vanillin (**3**) [10,11,35,36••,37]. Like many other aldehydes, vanillin plays an important role as a flavor and fragrance component of food as well as body-care and home-care products [38,39]. An elegant *de novo* route in yeasts utilizes *Ni*CAR for the synthesis of **3**. Undesired product removal by host enzymes was tackled by a selective gene knock out or *in situ* product removal by inserting a glycosyltransferase in order to mask **3** as vanillin β-D-glucoside (**4**) (Figure 3a) [36••]. Methyl branched aliphatic aldehydes, for example, impart the flavor impression of savory foods. *Mm*CAR was used in the form of a whole cell biocatalyst to prepare these target compounds from fungal lipid extracts with particularly prominent amounts of the respective methyl branched aliphatic acids [17]. Due to the demand for ATP and NADPH, microbial cells are currently considered to

Figure 1



Overview of substrate specificities of recombinant as well as purified CARs. The heat map represents CAR activities reported in the literature. Black indicates highest activities (k_{cat} 450 min^{-1}) and light grey (1) indicates lowest activities ($k_{cat} \leq 1$ min^{-1}). X represents reports of non-activity under the tested conditions. White: not described. For some substrate/CAR combinations, k_{cat} could not be calculated because reported data was based on endpoint measurements (e.g. [10,18,19]); the respective entries are estimations. In case of *Sr*CAR and *Mn*CAR, for example, kinetic data were available for benzoic acid. Activities for all other compounds were estimated based on reported relative yields and may be underestimated [14,15]. CAR types are indicated as roman numbers in front of their abbreviations in the headline.

Figure 2



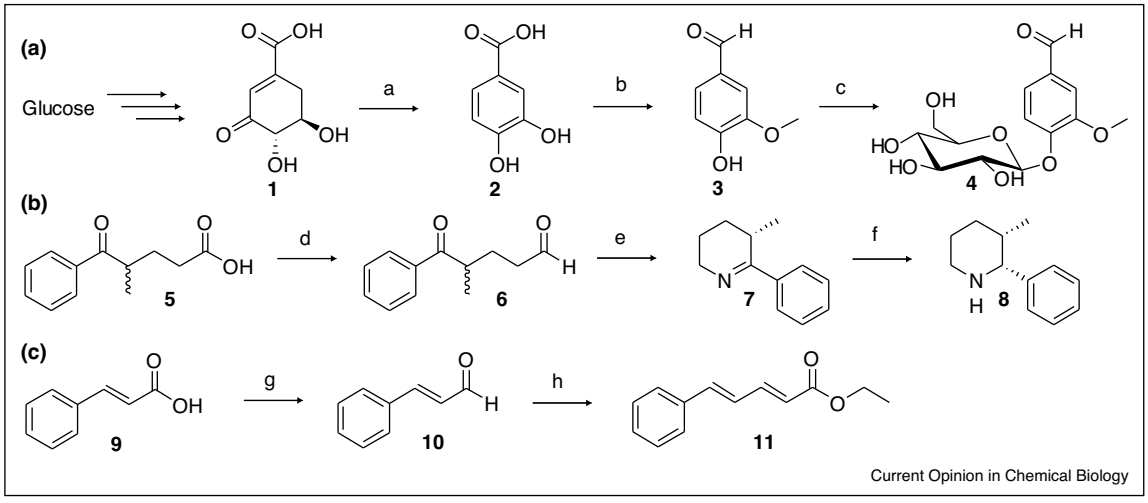
Phyre2 models of carboxylate reductases depict the three-domain architecture. **(a)** *Ni*CAR (type I CAR). The A-domain is depicted top left in pale cyan, the mobile C-terminal domain in salmon, the T-domain in deep teal, and the R-domain bottom right in blue; All templates <55% identity to *Ni*CAR. **(b)** *Nc*CAR (type III CAR). The A-domain is depicted top left in orange, the T-domain in dark grey, and the R-domain bottom right in medium grey. All templates < 25% identity to NcCAR; yellow serines in the T-domains represent the position of the phosphopantetheine attachment site. Green residues are conserved within the superfamily of CARs. Red residues mark catalytic residues (experimentally confirmed in *Nc*CAR, putative in *Ni*CAR).

be the most economic option for the use of CARs for preparative purposes [21]. These cells may be equipped with additional enzymes to immediately convert the reactive and sometimes toxic aldehyde species further. *Ni*CAR expressed in an engineered *E. coli* strain was coupled with a pyruvate decarboxylase to prepare L-phenylacetylcarbinol from the condensation product of benzaldehyde and pyruvate [37]. Another *in vivo* cascade took advantage of endogenous ADH activity of *E. coli* for the *Ni*CAR mediated preparation of the antioxidant 3-hydroxytyrosol [40]. The ability of *Mm*CAR to reduce aliphatic acids enabled the development of a multi enzyme cascade reaction starting from aliphatic keto acids. *Mm*CAR, in form of a whole cell biocatalyst, catalyzed the first step in this cascade, followed by enzymatic transamination, intramolecular cyclization and enantioselective enzymatic imine reduction to give chiral monosubstituted or disubstituted piperidines and pyrrolidines. A preparative example is shown in Figure 3b [18]. When biosynthetic pathways to fatty acids are coupled to CARs and decarbonylases, short to medium chain alkanes become accessible [6•,41,32]. Since these alkanes are bulk products in the fuel sector, it remains elusive if this technology may become competitive in future.

The selectivity of aldehyde preparation fuels the progress on CAR research. However, this product selectivity cannot yet be fully exploited. The reason is the co-factor demand, which is mostly fulfilled by cellular systems to avoid the high costs of these co-substrates. The use of cells, however, is accompanied by undesired reactions which are catalyzed by the organism's protein machinery. Using CARs in cellular systems leads to a trade-off between ATP/NADPH supply and product selectivity, because cellular background reactivities tend to remove reactive aldehyde species. Strain engineering was used to reduce overreduction of aromatic substrates by host proteins [42,43]. This strategy was extremely effective for particular substrates but much less for others [21]. *In vivo* cascades may be optimized by tuning the aldehyde amount to low levels, for example, by balancing the reaction rates of subsequent transformations [32]. Another strategy to optimize yields in cascades involving aldehyde intermediates is channelling [44], for example by scaffolding proteins [45] or using microcompartments [46]. Ultimately, the full potential of CARs product selectivity may also be exploited in cell free systems. To reach this goal, the recycling of NADPH and ATP *in vitro* need to be implemented.

Apart from the use of CARs in cascades *in vivo*, a one-pot chemoenzymatic *in vitro* cascade from 4-hydroxybenzoic acid to 4-hydroxybenzaldoxime was reported with *At*CAR [13]. Similarly, *Mn*CAR reduction provided aldehydes for a subsequent Wittig reaction to yield α,β-unsaturated esters, such as the ester derived from cinnamic acid

Figure 3



Selected examples of CAR mediated syntheses. **(a)** *De novo* synthetic pathway from glucose to vanillin in yeasts. (a) 3-Dehydroshikimic acid **1** is transformed to 3,4-dihydroxybenzoic acid **2** by 3-dehydroshikimate dehydratase. (b) *Ni*CAR and a O-methyltransferase reduce the acid and introduce a methyl group, resp., to give vanillin **3**. (c) Glucose is attached to **3** by a glycosyltransferase to yield **4** (adapted from Ref. [36••]). **(b)** Example for one-pot reaction cascade (d) ketoacid **5** is reduced by *Mm*CAR in the form of a whole cell biocatalyst to aldehyde **6**. (e) **6** is converted to an amine by an ω-transaminase and the product undergoes intramolecular cyclization to imine **7**. (f) (*S*) selective imine reductase affords (2R,3S)-**8** (adapted from Ref. [18]). **(c)** Example for sequential chemo-enzymatic route. (g) Cinnamic acid **9** is reduced to cinnamaldehyde **10** using *Mn*CAR *in vitro* with NADPH recycling. (h) **10** is coupled to ethylacrylate in a Wittig type reaction (adapted from Ref. [15]).

9 (Figure 3c) [15]. The *in vitro* use of CARs was so far hampered by a lack of efficient methods for the recycling of ATP, however, recent progress in ATP recycling will certainly facilitate the development of *in vitro* systems in the near future [47].

Conclusions

A current trend is to generate diversity in the CAR toolbox. The goals are to identify and characterize CARs with outstanding properties such as thermostability or high activities for particular substrates. There is a need to understand more about the structure–function relationship of CARs to allow focused engineering of known protein scaffolds towards these goals.

CARs reduce carboxylates to aldehydes and do not reduce the aldehyde any further, although aldehydes are significantly more prone to reduction than acids. From the application viewpoint, this product selectivity of carboxylate reducing enzymes is a highly appealing feature, which can, however, not yet be fully exploited. The demand for ATP and NADPH is mostly fulfilled by cellular systems to save costs, but cellular background reactivities deminish theoretically perfect aldehyde levels by side reactions. *In vitro* applications may become a solution to this dilemma.

It seems likely that CAR enzymes will find further applications in the production of volatiles for the flavor and fragrance sector. Another trend and major field of application is the integration of CARs in cascade reactions with aldehydes as the key intermediate. Hence, CARs can be expected to contribute in various reaction cascades towards pharmaceutical products and fine chemicals in the near future.

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