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Random Mutagenesis-Driven Improvement of Carboxylate Reductase Activity using an Amino Benzamidoxime-Mediated High-Throughput Assay

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Abstract: Carboxylic acid reductases (CARs) catalyze the direct adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) dependent reduction of carboxylic acids to their corresponding aldehydes. The identification and improvement of CARs by protein engineering is, however, severely limited by the lack of fast and generic methods to quantify aldehydes. Within this study, we applied a convenient high-throughput assay (HTA) based on amino benzamidoxime (ABAO) that allows the substrate-independent and chemoselective quantification of aldehydes. Random mutagenesis of the well-known CAR from *Nocardia iowensis* (CAR_{Ni}) to improve its activity for sterically demanding 2-substituted benzoic acid derivatives was conducted in a K_M-dependent fashion, and the HTA applied in the presence of microbial cells. The study identified a hot spot in the active site of CAR_{Ni} that increased the affinity to 2-methoxybenzoic acid 9-fold upon mutation from glutamine to proline (Q283P). The catalytic performance of $CAR_{NiQ283P}$ appeared to be significantly improved also for other substrates such as 2-substituted (2-Cl, 2-Br) as well as 3- and 4-substituted benzoic acids (3-OMe, 4-OMe), and even aliphatic octanoic acid.

Keywords: carboxylic acid reductase (CAR); aldehyde; amino benzamidoxime; mutagenesis; high-throughput screening; carboxylic acids

Introduction

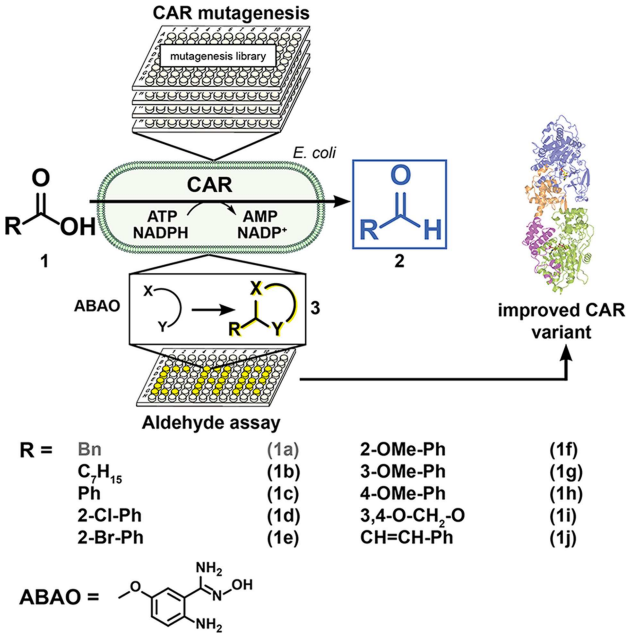
Aldehydes are important reactive handles for the synthesis of complex molecules for fine chemicals and pharmaceutical ingredients.[1] They also play a prominent role as ingredients of fragrances in perfumery, home- and body care products, and as flavors in the food sector.[2] Aldehydes can be synthesized by ozonolysis of alkenes, by oxidation of primary alcohols, or the reduction of activated carboxylic acid derivatives (e.g. halides and esters). A very appealing approach is the selective and direct reduction of the stable carboxylic acid moiety towards the highly reactive aldehyde species. The direct reduction in organic solvent by hydrosilylation is promising,[3] however, a typical drawback of reductive methods is the over-reduction of the desired aldehyde to the respective alcohols.[4]

Carboxylic acid reductases (CARs) are an emerging class of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) dependent enzymes.[5] They show remarkably broad

substrate scope, and display exceptionally high intrinsic chemoselectivity leading solely to the aldehyde. CARs are structurally flexible, post-translationally modified three-domain proteins and approximately 100–130 kDa in size. Recently, the groups of Turner and Leys elucidated the structure of different bacterial CAR domains individually[6] and provided further evidence to their mode of action. ATP activates the carboxylic acid via a reactive AMP anhydride intermediate, which is then attacked by the phosphopantetheine thiol nucleophile. The resulting thioester is reduced to the aldehyde by concomitant hydride transfer from NADPH to ultimately release the product aldehyde (Supporting Information, Figure S1[7]).[6,8] Their size, their need for post-translational modification and their cofactor requirement render CARs a somewhat complex and synthetically still underrepresented enzyme class. Few sequences of CARs have been elucidated and synthetically explored by heterologous expression in *Escherichia coli* (*E. coli*).[6,8–9] The necessity of stoichiometric amounts of ATP and NADPH for aldehyde formation requires a cofactor recycling system or, alternatively, the use of whole cells providing the cofactors via the cellular metabolism.[8–9,10]

The potential of CAR-mediated aldehyde synthesis has been recognized by industry.[11] Further successful applications, however, are hampered by the discovery of new enzymes with characteristics compatible to process conditions or the engineering of existing CAR proteins to achieve desired metrics, e. g. productivity. With the structural data of CARs in hand,[6] rational protein engineering may now be performed on subgroup I CARs.[12] Due to low sequence identities (< 26%) to other subgroups, the majority of CAR engineering endeavors still rely on random mutagenesis. To enable efficient identification of new CAR variants, we developed an amino benzamidoxime (ABAO)-based assay for screening mutant libraries in a whole cell system.[13] CARs may thus be developed faster into a broadly applicable and widely used enzyme class.

Herein, we report a mutagenesis study applied to the CAR from *Nocardia iowensis* (CAR_{Ni}, Q6RKB1.1), the perhaps best studied CAR up to date,[8] using the ABAO-assay as the key tool for aldehyde detection (Scheme 1).



Scheme 1. High-throughput assay (HTA) for structurally diverse aldehyde detection and application on aldehyde forming whole cell biocatalysts.

Results and Discussion

As described in our recent publication,[13] aldehyde formation by CAR_{Ni} was investigated using an engineered E. coli strain with Reduced Aldehyde Reduction capability.[14] Product yields determined by the ABAO-assay were confirmed by GC and HPLC measurements and indicated the assay's utility as a quantitative analytical tool to be used in the presence of resting cells. Sample preparation steps such as centrifugation, dilution, pH adjustment and on-plate calibration were adapted to fit the needs of a high-throughput validation of CAR_{Ni} variants.[13]

Benzoic acid derivatives are reduced well by CAR_{Ni}, with the exception of derivatives with steric bulk in position 2. In previous literature it was postulated that the steric hindrance in 2-position either prohibits substrate binding or slows down the AMP anhydride formation tremendously, as determined by initial rate measurements.[15] In our hands, benzoic acid (**1c**) was reduced to good yields (66%) of aldehyde **2c** after 5 h, whereas 2-methoxy benzoic acid (**1f**) resulted in only 11% of the desired aldehyde, indicating that substrate binding is not strictly prohibited. A similar result was observed with 2-bromo benzoic acid (**1e**), hence indicating the principle ability of CAR_{Ni} to reduce such substrates.

The poor yields of 2-substituted benzoic acid derivatives motivated to gain deeper mechanistic insights and prompted us to investigate the kinetic parameters for 2-methoxy benzoic acid (**1f**). Whereas k_{cat} (0.234 s^{-1}) was moderate in comparison to other CAR_{Ni} substrates, K_M was significantly higher (K_M = 64 mM).[15] This low affinity impedes efficient whole cell biotransformations and intrigued us to design a protocol with sub-K_M-substrate concentration (25-fold below the actual K_M-value) to allow for selection of variants with improved substrate affinity. 2-Methoxybenzoic acid (**1f**) was the target substrate for the following mutagenesis study. Its corresponding alde-

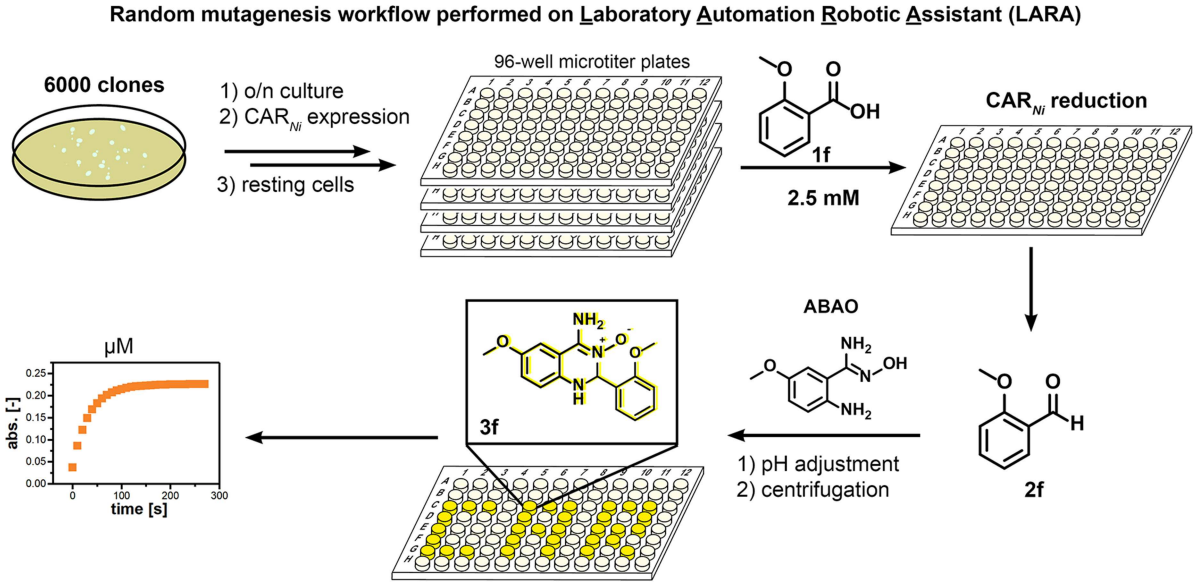


Figure 1. Flow scheme of HTA application for screening of CAR$_{Ni}$ mutation libraries. Inoculation of 96-well microtiterplates (expression plates) by a picking robot (upper left corner) represents the first step. Expressed cells were harvested and used for the bioconversion of **1 f** to **2 f** in the same plates (upper right corner). After addition of **ABAO** in buffer (lower right corner), cells were centrifuged and the supernatant was transferred to a new set of 96-well microtiter plates. **3 f** in the supernatant was subsequently measured by UV measurements (lower left corner) and used to quantify conversions.

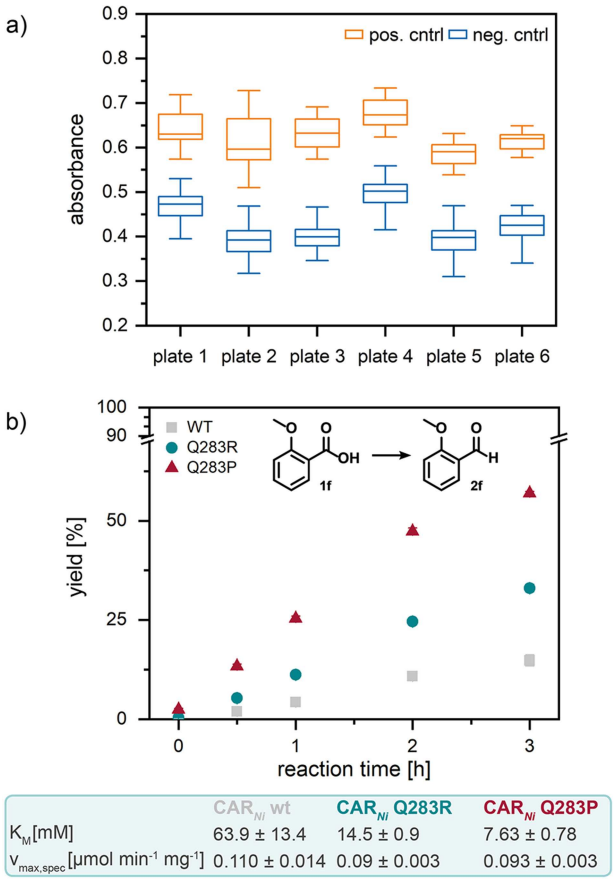
hyde **2 f** was found to be an active compound against food mites.[16]

All EC 1.2.1.30 CARs known to date share the same domain architecture, an adenylating domain (A-domain) for substrate activation, a reductase domain (R-domain) for actual reduction of the substrate, and a linker domain.[7,12]

Essential for activity is the post-translational modification of the linker domain by the transfer of phosphopantetheine from Coenzyme A to a conserved serine.[7] We performed directed evolution through random mutagenesis on basis of the plasmid pET-Duet1_*Ec*PPTase_*Ni*CAR expressing the CAR$_{Ni}$ together with a phosphopantetheinyl transferase (PPTase) from *E. coli* for post-translational activation. Specifically, two selected regions in the A- and the R-domain were targeted, respectively (Supporting Information, Figure S2). We aimed at an average final amino acid exchange of approximately 1 per mutated gene. For each library, 12 individual clones were sequenced (Supporting Information, Chapter 3.1). The average amino acid exchange rate was 0.92 and 1.2 per protein, for the A-domain and the R-domain, respectively. The obtained plasmid libraries isolated from *E. coli* NEB5α were used for transformation of *E. coli* RARE.[14]

The workflow to screen for improved mutants is summarized in Figure 1. Briefly, single colony mutants, plated on standard agar-plates, were transferred into 96-well microtiter plates. Cultivation and expression of all clones was performed in an autoinduction medium (LB-5052).[17] Subsequently, cells were harvested and 2.5 mM of **1 f** was added in M9 medium without further nitrogen source (see Supporting Information, Chapter 2). After 5 h, the pH value was adjusted to 4.5, **ABAO** was added and the cells were removed by centrifugation. Reaction progress was monitored by UV, as depicted in Figure 1. Screening of the large scale mutant panel (6,000 mutants) was performed on the robotic platform "LARA" (Laboratory Automation Robotic Assistant http://lara.uni-greifswald.de).[18] Screening of the saturation library including the on-plate calibration was performed manually (Supporting Information, Chapter 3.3.3). The symbiotic combination of assay and robotic platform enabled screening and analysis of approximately 4500 clones of the A-domain and 1500 clones of the R-domain within seven days. Wells displaying no growth were excluded from analysis.

The accuracy of the ABAO-assay was confirmed by statistical evaluation of selected positive and negative control experiments in the presence of living cells (Figure 2a). Control wells on each plate (positive and negative), and a calibration with the desired aldehyde product ensures inter-plate comparability. Hits from the original screening were subjected to manual rescreening. One outstanding hit variant showed approximately a five-fold lowered K_m-value by one amino acid exchange from glutamine to arginine (Q283R) as compared to CAR$_{Ni}$ wild-type. Position 283 is in approximately 10 Å distance to the substrate binding site. The mutation was therefore unlikely creating more space for the bulky substituent.



	CAR_{Ni} wt	CAR_{Ni} Q283R	CAR_{Ni} Q283P
K_M[mM]	63.9 ± 13.4	14.5 ± 0.9	7.63 ± 0.78
$v_{max,spec}$[μmol min^{-1} mg^{-1}]	0.110 ± 0.014	0.09 ± 0.003	0.093 ± 0.003

Figure 2. a) Absorbance dependency of individual well-plates. Absolute values are drifting but relative differences between false negative and false positives maintained constant. b) top: Yields of **2 f** in whole cell biotransformations (HPLC analysis), bottom: Kinetic parameters of CAR_{Ni} wt and variants for **1 f** reduction (Supporting Information, Figure S9–11).

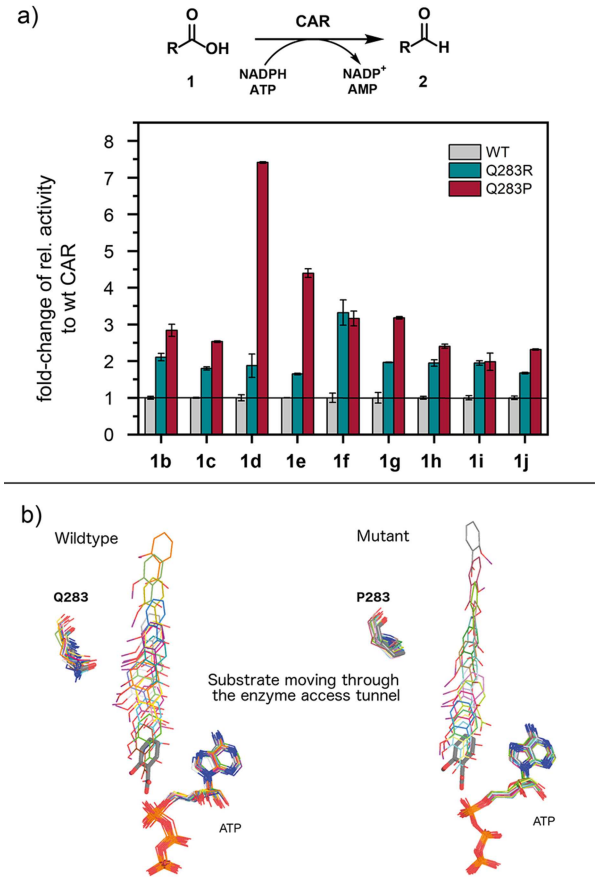


Figure 3. a) Relative fold-change of specific activities of Q283R and Q283P variants compared to CAR_{Ni} wt determined by NADPH depletion (Supporting Information, chapter 3.3.3, for absolute activities see Figure S12). b) Residue 283 lines the active site access tunnel as revealed by steered molecular dynamics simulations. The amide side chain of Q283 contacts the methoxy substituent of **1 f** whereas in the Q283P variant, no contact of the amino acid is seen. The modeled ATP-bound form of CAR_{Ni} and its Q283P variant is based on the structure 5MSD.[6]

To unravel the impact of position 283, it was subjected to site saturation mutagenesis (Supporting Information, Chapter 3.2). The respective saturation library was analyzed by both the ABAO-assay including on-plate calibration and HPLC measurements (Supporting Information, Chapter 3.3.3). All Q283X variants displayed decent to excellent activity (Supporting Information, Figure S11). Both methods revealed that the variant with proline on the position 283 (Q283P) as the most active candidate for **1 f** reduction (Supporting Information, Figure S7).

Wild-type CAR_{Ni} and the Q283 variants were purified and analyzed *in vitro* (Figure 2b, bottom, and Figure 3a). The A-domain variant Q283R (glutamine to arginine) showed approximately two-fold higher aldehyde yield in biotransformation experiments using whole cells (Figure 2b, top). The improvement can be attributed to 4-fold higher affinity ($K_M = 14$ mM) towards **1 f** at unchanged v_{max} compared to the wild-type (wt) ($K_M = 64$ mM, Figure 2b, bottom). With the single amino acid exchange for proline, the affinity for **1 f** increased 9-fold ($K_M = 7.6$ mM) in comparison to wild type CAR_{Ni}, translating to 4-fold higher yield. This positive effect was also observed for other 2-substituted benzoic acids: in fact, $CAR_{Ni Q283P}$ showed increased activity for all tested substrates as shown in Figure 3a and Supporting Information, Figure S12. Phenylacetic acid (**1 a**) only served as an assay control substrate and is not included in Figure 3a.

To gain deeper insights into the observed phenomenon, we applied steered molecular dynamics simulations,[19] where **1 f** was retracted from the active site by applying a directed constant acceleration during simulation (Figure 3b, snapshots overlay; Supporting Information, Chapter 3.5). In these simulations, the methoxy substituent of **1 f** contacts the amide of the glutamine side chain (Q283) in several snapshots. This

is not observed in the proline-substituted variant. We hypothesize that position 283 effects the dynamics of substrate entry towards the active site.

Conclusion

In summary, we applied the ABAO-assay in the presence of living cells for a mutational study. 2-Methoxybenzoic acid (**1f**) – a reported non-substrate of CARs[15] – was used as a starting point to engineer more efficient CAR_{Ni} variants. Improved variants showed comparable k_{cat} values, whereas the affinity (K_M) towards **1f** was improved. A mutation from glutamine to arginine (Q283R, A-domain) in position 283 resulted in a 4-fold increase in affinity. A proline in this position further decreased K_M (9-fold) and increased the yield of **2f** to 56% within 3 hours with proline at position 283 as determined by *in vivo* reactions. A similar trend was observed for other 2-substituted (2-Cl, 2-Br) as well as 3- and 4-substituted benzoic acids (3-OMe, 4-OMe), and aliphatic octanoic acid. The screening of carboxylate reductase libraries composed of thousands of clones in a reasonable timeframe can now be accomplished for the first time in a manual setup or assisted by robotic platforms.

Experimental Section

For detailed experimental procedures, primer lists and analytical methods see Supporting information. Phenylacetic acid (**1a**) was used as the substrate for assay control. All dihydroquinazoline products **3a–j** were synthesized on preparative scale and analyzed by NMR.[13]

Using the ABAO-Assay to Screen Resting Cell Biocatalysts in High-Throughput Format

96-deep well plates (DWP) filled with 800 µL initial growth medium (LB 0.8G)[17] containing 100 µg/mL ampicillin were inoculated with colonies of CAR_{Ni} in *E. coli* MG1655 RARE (DE3). Specifically, 3 wells were used for negative controls (e.g. pETDuet:*Ec*PPTase*Ni*CARΔA, an A-domain deletion variant which lacks a stretch of the A-domain, 3 wells sterile controls and 6 wells for positive controls (pETDuet:*Ec*PPTase-*Ni*CAR). 18 additional wells were inoculated with pETDuet:*Ec*PPTase*Ni*CARΔA for on-plate calibration. These master plates were incubated over night at 37 °C and 1000 rpm on a Heidolph Titramax 1000 until cells were in the exponential growth phase. Freshly prepared 96-DWPs filled with 800 µL LB-5052[17] containing 50 µg/mL ampicillin were inoculated from the master plates by a microplate replicator. The copy step from the master plate to the expression plate ensured the comparable starting point for expression as described by Dörr *et al.*.[18] Expression plates were incubated for 4 h at 37 °C and 1000 rpm on a Heidolph Titramax 1000, subsequently the temperature was lowered to 20 °C for expression with the same shaking speed. After 24 h total time for growth and expression, cells were harvested by centrifugation at 3220 × g for 20 min and the supernatant was removed by decantation. The remaining cell pellets were resuspended in 400 µL of conversion buffer (M9 medium without ammonium chloride, containing 0.8% glucose and 2.5 mM of **1f**, Supporting Information, chapter 2). Cells in 'on-plate calibration wells' were resuspended in buffer with **2f** in a concentration range between 0.1 and 1.5 mM). The plates were incubated at 25 °C and 1000 rpm for 5 h. Next, 400 µL of **ABAO**-solution (sodium acetate buffer 100 mM, pH 4.5 containing 5% DMSO and 10 mM of **ABAO**) were added and the plates were centrifuged at 3220 x g for 30 min. The supernatants (150 µL) were transferred to a fresh plate via multichannel pipette and analyzed at 380 nm. On-plate calibration curves were used to calculate the amount of **2f** produced by each clone.

Whole Cell Biotransformation

Biotransformations were conducted in 100 ml flasks in a volume of 10 ml. Resting cells were prepared as described in Supporting information chapter 2.3.1. The biotransformation was performed at an OD_{600} of 10 in M9 medium (no nitrogen, 0.8% glucose) with a carboxylic acid concentration (**1f**) of 2.5 mM. The flaks were shaken at 100 rpm at 25 °C in a Multitron shaker and samples were taken after 0.5, 1, 2 and 3 h and analyzed by HPLC-UV. Reactions were performed in triplicates.

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