Awakening the Sleeping Carboxylase Function of Enzymes: Engineering the Natural CO₂-Binding Potential of Reductases

Iria Bernhardsgrütter,† Kristina Schell,‡ Dominik M. Peter,† Farshad Borjian,‡ David Adrian Saez,§ Esteban Vöhringer-Martínez,∥ and Tobias J. Erb*†,∥

†Department of Biochemistry and Synthetic Metabolism, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Straße 10, D-35043 Marburg, Germany
‡Institute for Molecular Microbiology and Biotechnology, University of Münster, Corrensstr. 3, D-48149 Münster, Germany
§Departamento de Físico Química, Facultad de Ciencias Químicas, Universidad de Concepción, 1290 Concepción, Chile
∥LOEWE Center for Synthetic Microbiology (Synmikro), Karl-von-Frisch-Straße 16, D-35043 Marburg, Germany

Supporting Information

ABSTRACT: Developing new carbon dioxide (CO₂) fixing enzymes is a prerequisite to create new biocatalysts for diverse applications in chemistry, biotechnology and synthetic biology. Here we used bioinformatics to identify a “sleeping carboxylase function” in the superfamily of medium-chain dehydrogenases/reductases (MDR), i.e. enzymes that possess a low carboxylation side activity next to their original enzyme reaction. We show that propionyl-CoA synthase from Erythrobacter sp. NAP1, as well as an acryl-CoA reductase from Nitrosopumilus maritimus possess carboxylation yields of 3 ± 1 and 4.5 ± 0.9%. We use rational design to engineer these enzymes further into carboxylases by increasing interactions of the proteins with CO₂ and suppressing diffusion of water to the active site. The engineered carboxylases show improved CO₂-binding and kinetic parameters comparable to naturally existing CO₂-fixing enzymes. Our results provide a strategy to develop novel CO₂-fixing enzymes and shed light on the emergence of natural carboxylases during evolution.

To harvest atmospheric CO₂ as a sustainable carbon source for (bio)catalytic and (bio)technological applications,¹−³ it is necessary to extend the repertoire of CO₂-fixing reactions. One possibility is to engineer a carboxylation function into the scaffold of non-CO₂-fixing enzymes. Generally, the interaction of CO₂ with proteins is poorly understood.⁶ However, for enoyl-CoA carboxylase/reductase from Kitasatospora setae (ECRKs), four conserved amino acids that form a CO₂-binding pocket at the active site were described recently⁷ (Figure 1a). These four amino acids anchor and position the CO₂ molecule during catalysis, in which a reactive enolate is formed that attacks the CO₂.⁸

To identify enzyme scaffolds capable of binding CO₂ beyond the ECR enzyme family, we searched homologues of the MDR superfamily for the CO₂-binding motif. Our search revealed two enzyme families that show the potential to bind CO₂, the propionyl-CoA synthase (PCS) and an archaeal enoyl-CoA reductase (AER) family (Figure 1b). The PCS family clusters closely to ECRs and shows a fully conserved CO₂-binding motif across individual family members (Figure S1). The AER family is more distantly related to the ECR family, and selected homologues only contain one or two of the four conserved residues of the CO₂-binding pocket (Figure S2). We decided to test selected members of these enzyme families in their CO₂-fixing capabilities.

PCS is a three-domain fusion enzyme that catalyzes the overall conversion of 3-hydroxypropionate to propionyl-CoA¹⁰ (Figure 2a). The enzyme forms a central reaction chamber, in which three subsequent reactions take place in a synchronized fashion.¹¹ When we assayed PCS from Erythrobacter sp. NAP1, PCSEN, at 4.4 mM dissolved CO₂, we detected minor amounts of methylmalonyl-CoA besides the main product propionyl-CoA. Incorporation of ¹³CO₂-label confirmed the latent carboxylation activity of PCSEN (Figure 2b). Notably, the carboxylation function was not limited to the Erythrobacter enzyme, but was also detected with PCS from Chloroflexus aurantiacus (PCSₐ Table S1).

Received: April 10, 2019 Published: June 12, 2019
The last reaction in the three-reaction sequence of PCS is the reduction of acrylyl-CoA to propionyl-CoA, catalyzed by a reductase domain harboring the CO₂-binding motif (Figure 2a,c). We directly tested the reductase domain for carboxylation activity with an E1027Q variant of PCS EN (PCSEN\(_{ΔDH}\)) that is unable to generate acrylyl-CoA. When PCSEN\(_{ΔDH}\) was provided with external acrylyl-CoA and 4.4 mM dissolved CO₂, the enzyme showed a carboxylation yield (defined as percentage yield of carboxylated product compared with total product formed, including reduced side product) of 3 ± 1% (Table 1). This showed that the reductase domain is able to carbonate acryl-CoA directly.

To improve further the carboxylation efficiency of PCSEN, we compared the active site of PCSEN (PDB: 6EQO) with ECRKs. While the NADPH binding site, as well as the four CO₂-binding pocket residues are structurally conserved (Figure 2c), we noticed differences in the second shell of the active site. ECRKs features a small hydrophilic residue (Thr82), which interacts with Asn81 that stabilizes CO₂ through its carboxyamide NH₂ group. The corresponding residue in PCSEN is occupied by an aspartate (Asp1302). Molecular dynamics (MD) simulations demonstrated that Asp1302 in PCSEN forms a strong anionic hydrogen bond to the carboxamide NH₂ group of Asn1301 (Figures 3a and S5), locking Asn1301 in a position which prevents interactions with CO₂. This finding is in line with the fact that we could not determine an apparent \( K_M \) for CO₂ with PCSEN\(_{ΔDH}\) and that

![Figure 2](image1.png)

**Figure 2.** PCSEN possesses a “sleeping carboxylase function”. (a) Reaction sequence of PCS. PCS natively catalyzes the conversion of 3-hydroxypropionate into propionyl-CoA (solid lines) and possesses a low carboxylation activity yielding methylmalonyl-CoA (dashed line). (b) High-performance liquid chromatography-mass spectrometry traces of the PCSEN overall reaction showing 3-hydroxypropionyl-CoA, propionyl-CoA and (3-\(^12/13\)C)-methylmalonyl-CoA at \(m/z\) 840.14, 824.15 and 868.13/869.13, respectively. Methylmalonyl-CoA is only detected in the presence of \(^{12/13}\)CO₂ (provided as bicarbonate). Data represent an individual experiment with two replicates. (c) Active site of PCSEN reductase domain (cyan, PDB: 4EQO) and ECRKs (blue), both cocrystallized with NADP⁺. Acrylyl-CoA and CO₂ are modeled into the active site. WebLogo-Illustration of conserved active site residues using 129 PCS and 29 ECR sequences. Numbering according to PCS EN or ECRKs, respectively.

![Figure 3](image2.png)

**Figure 3.** Directed mutagenesis to exploit the carboxylation activity of PCSEN. (a) Representative snapshot from the MD simulation of the active site in wild type PCSEN. (b) Active site model of PCSEN D1302S to unlock Asn1301. (c) Active site of wild type PCSEN. (d) Active site model of PCSEN T1753M to restrict water access to the active site. Acryl-CoA and CO₂ were modeled into the active site.

| Table 1. Reaction Parameters and Carboxylation Yield for the Reductase Domain of Different PCSEN Variants\(^a\) |
|-------------------|-------------------|-------------------|-------------------|
| PCSEN variant     | \( k_{cat} \) (s\(^{-1}\)) at 4.4 mM CO₂ | \( K_M^{acryl-CoA} \) (mM) | % carboxylation at 4.4 mM CO₂ | \( K_M^{CO_2} \) (mM) |
| PCSEN\(_{ΔDH}\) WT | 7.4 ± 1.0         | 0.014 ± 0.002     | 3 ± 1              | n.m.               |
| PCSEN\(_{ΔDH}\) D1302S | 1.77 ± 0.09     | 0.027 ± 0.003     | 20.9 ± 0.7         | 27 ± 5             |
| PCSEN\(_{ΔDH}\) T1753M | 6.5 ± 0.6       | 0.0197 ± 0.0012   | 10 ± 2             | n.m.               |
| PCSEN\(_{ΔDH}\) D1302S T1753M | 0.46 ± 0.03   | 0.026 ± 0.003     | 69 ± 3             | 26 ± 5             |

\( k_{cat} \) shows combined reduction and carboxylation activity. \( K_M \) values were determined from a Michaelis–Menten fit of at least 18 data points, with fixed acryl-CoA concentrations for \( K_M^{CO_2} \) (Figures S3 and S4, Table S2 for \( k_{cat} \) values). Carboxylation yields are calculated from mean carboxylation yields over five time points in three replicates. Data are mean ± s.d. CO₂ concentrations were calculated. n.m., not measurable.

\(^a\) DOI: 10.1021/jacs.9b02043
J. Am. Chem. Soc. 2019, 141, 9778–9782
replacing Asn1301 by an aspartate abolished carboxylation activity.

We aimed at unlocking Asn1301 from its fixed position by replacing Asp1302 with different small hydrophilic residues. PCS_

\[ \text{combining the D1302S with the T1573M mutation, the replacing Asp1302 with di} \]

To increase the carboxylation e

\[ \text{combining the D1302S with the T1573M mutation, the replacing Asp1302 with di} \]

In PCSEN, this residue is a threonine, which presumably allows water to enter the active site and displace the CO2 molecule. In PCSEN, this residue is a threonine, which presumably allows water to enter the active site and displace the CO2 molecule.27 In ECRKs, a conserved methionine (Met356) restricts access of water to the CO2-binding pocket.28 When we introduced the methionine in PCSEN (PCSEN_

\[ \text{combining the D1302S with the T1573M mutation, the replacing Asp1302 with di} \]

Our engineering e

\[ \text{combining the D1302S with the T1573M mutation, the replacing Asp1302 with di} \]

In conclusion, we successfully reshaped the energy landscape of acrylyl-CoA reductases from the thermodynamically favored product propionyl-CoA (\( \Delta\text{G}^{\text{a}} \approx -63 \text{ kJ/mol} \)) to the disfavored methylmalonyl-CoA (\( \Delta\text{G}^{\text{a}} \approx -43 \text{ kJ/mol} \)). Our engineering efforts show that improving CO2-binding (reduced energy barrier for carboxylation) and minimizing side reaction with water (increased energy barrier for reduction) are both required to establish a carboxylation activity in the scaffold of different reductases. This is in line with the idea that in catalysis stabilization of favorable transition states ("positive catalysis") and destabilization of unwanted transition states ("negative catalysis") are both important,24–26 as further supported by the finding that suppression of competing protonation side reactions is essential for efficient CO2-fixation.

Table 2. Reaction Parameters and Carboxylation Yield for AER_

\[ \text{combining the D1302S with the T1573M mutation, the replacing Asp1302 with di} \]

In conclusion, we successfully reshaped the energy landscape of acrylyl-CoA reductases from the thermodynamically favored product propionyl-CoA (\( \Delta\text{G}^{\text{a}} \approx -63 \text{ kJ/mol} \)) to the disfavored methylmalonyl-CoA (\( \Delta\text{G}^{\text{a}} \approx -43 \text{ kJ/mol} \)). Our engineering efforts show that improving CO2-binding (reduced energy barrier for carboxylation) and minimizing side reaction with water (increased energy barrier for reduction) are both required to establish a carboxylation activity in the scaffold of different reductases. This is in line with the idea that in catalysis stabilization of favorable transition states ("positive catalysis") and destabilization of unwanted transition states ("negative catalysis") are both important,24–26 as further supported by the finding that suppression of competing protonation side reactions is essential for efficient CO2-fixation.

9780

DOI: 10.1021/jacs.9b03431

J. Am. Chem. Soc. 2019, 141, 9778–9782
in ECR<sub>NC</sub> and 2-ketopropyl coenzyme M oxidoreductase/carboxylase.<sup>14–16</sup> On a broader picture, our findings also raise questions about the emergence of natural carboxylases. How did carboxylation functions naturally evolve in the scaffold of proteins, such as Rubisco or ECR? It has been suggested that these enzymes originated from non-CO<sub>2</sub>-fixing ancestors.<sup>27,28</sup> Our data provides experimental evidence for this evolutionary scenario by demonstrating that the MDR superfamily, to which ECR belongs, naturally possesses the capacity to interact with the CO<sub>2</sub>-molecule. It apparently takes only a few mutations to transform latent carboxylases that convert CO<sub>2</sub> at low efficiency and nonphysiological CO<sub>2</sub> concentrations into decent CO<sub>2</sub>-fixing enzymes.

Another apparent question is why PCS and AER would possess a "sleeping carboxylase function"? One explanation might be that the latent carboxylation activity was selected for. PCS operates in the 3-hydroxypropionate bicycle in C. aurantiacus and a modified version thereof in Erythrobacter sp. NAP1 (Figure S7a),<sup>29,30</sup> while AER<sub>Nm</sub> presumably works in the 3-hydroxypropionate/4-hydroxybutyrate cycle in N. marinus (Figure S7b).<sup>18</sup> Bioenergetic considerations suggest that even a low carboxylation activity would increase biomass yield (Supporting Information III). In summary, our proof-of-principle study demonstrates that it is possible to exploit the active site of reductases to create novel carboxylases. This opens the possibility for the future engineering of novel CO<sub>2</sub>-fixing enzymes that could find application in biocatalysis and synthetic biology (e.g., in artificial pathways for the conversion of CO<sub>2</sub>).<sup>31,52</sup>

## ASSOCIATED CONTENT

### Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b03431.
Experimental details, biochemical enzyme characterization, compound synthesis, analytical HPLC methods, bioinformatics methods, supporting figures and tables (PDF)

## AUTHOR INFORMATION

### Corresponding Author
*toerb@mpi-marburg.mpg.de*

### ORCID
David Adrian Saez: 0000-0001-5555-8720
Esteban Vöhringer-Martinez: 0000-0003-1785-4558
Tobias J. Erb: 0000-0003-3685-0894

### Notes
The authors declare no competing financial interest.

## ACKNOWLEDGMENTS
We thank Gabriele Stöffel for his input on the mechanism of CO<sub>2</sub>-binding in ECRs and Niña Socorro Cortina for operating the hrLC-MS. The work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported under Contract No. DE-AC02-05CH11231. This work was funded by the Deutsche Forschungsgemeinschaft through Collaborative Research Centre SFB 987, the European Research Council (ERC 637675 "SYBORG"), the Gebert-Rüf-Stiftung (GRS 062-12), the Max-Planck-Society Partnerprogram and the Max-Planck-Society.

## REFERENCES

1. Glueck, S. M.; Gümüs, S.; Fabian, W. M.; Faber, K. Biocatalytic carboxylation. *Chem. Soc. Rev.* 2010, 39, 313–328.
2. Martin, J.; Eisoldt, L.; Skerra, A. Fixation of gaseous CO<sub>2</sub> by reversing a decarboxylase for the biocatalytic synthesis of the essential amino acid L-methionine. *Nat. Catal.* 2018, 1, 555.
3. Plasch, K.; Hofer, G.; Keller, W.; Hay, S.; Heyes, D. J.; Dennig, A.; Glueck, S. M.; Faber, K. Pressurized CO<sub>2</sub> as a carboxylating agent for the biocatalytic ortho-carboxylation of resorcinol. *Green Chem.* 2018, 20, 1754–1759.
4. Peter, D. M.; Schada von Borzyskowsky, L.; Kiefer, P.; Christen, P.; Vorholt, J. A.; Erb, T. J. Screening and Engineering the Synthetic Potential of Carboxylating Reductases from Central Metabolism and Polyketide Biosynthesis. *Angew. Chem., Int. Ed.* 2015, 54, 13457–13461.
5. Zhang, L.; Morii, T.; Zheng, Q.; Awakawa, T.; Yan, Y.; Liu, W.; Abe, I. Rational Control of Polyketide Extender Units by Structure-Based Engineering of a Crotonyl-CoA Carboxylase/Reductase in Antimycin Biosynthesis. *Angew. Chem., Int. Ed.* 2015, 54, 13462–13465.
6. Cundari, T. R.; Wilson, A. K.; Drummond, M. L.; Gonzalez, H. E.; Jorgensen, K. R.; Payne, S.; Braunfeld, J.; De Jesus, M.; Johnson, V. M. Co<sub>2</sub>-formicats: how do proteins bind carbon dioxide? *J. Chem. Inf. Model.* 2009, 49, 2111–2115.
7. Stoffel, G. M. M.; Saez, D. A.; DeMirchi, H.; Vögel, B., Yao, Y., Zarzyczi, J., Yoshikuni, Y., Wakatsuki, S., Vöhringer-Martinez, E.; Erb, T. J. Four amino acids define the CO<sub>2</sub>-binding pocket of enoyl-CoA carboxylases/reductases. *Proc. Natl. Acad. Sci. U.S.A.* 2019, in press.
8. Rosenthal, R. G.; Ebert, M.-Ö.; Kiefer, P.; Peter, D. M.; Vorholt, J. A.; Erb, T. J. Direct evidence for a covalent ene adduct intermediate in NAD (P) H-dependent enzymes. *Nat. Chem. Biol.* 2014, 10, 50.
9. Hedlund, J.; Jörnvall, H.; Persson, B. Subdivision of the MDR superfamily of medium-chain dehydrogenases/reductases through iterative hidden Markov model refinement. *BMC Bioinf.* 2010, 11, 534.
10. Alber, B. E.; Fuchs, G. Propionyl-coenzyme A synthase from *Chloroflexus aurantiacus*, a key enzyme of the 3-hydroxypropionate cycle for autotrophic CO<sub>2</sub> fixation. *J. Biol. Chem.* 2002, 277, 12137–12143.
11. Bernhardsgrüter, I.; Vögel, B.; Wagner, T.; Peter, D. M.; Cortina, N. S.; Kahnt, J.; Bange, G.; Engelbrecht, S.; Girard, E.; Riöbé, F.; et al. The multicatalytic compartment of propionyl-CoA synthase sequencers a toxic metabolite. *Nat. Chem. Biol.* 2018, 14, 1127.
12. Crooks, G. E.; Hon, G.; Chandonia, J.-M.; Brenner, S. E. WebLogo: a sequence logo generator. *Genome Res.* 2004, 14, 1188–1190.
13. Schneider, T. D.; Stephens, R. M. Sequence logos: a new way to display consensus sequences. *Nucleic Acids Res.* 1990, 18, 6097–6100.
14. Pandey, A.; Mulder, D.; Ensign, S.; Peters, J. Structural basis for carbon dioxide binding by 2-ketopropyl coenzyme M oxidoreductase/carboxylase. *FEBS Lett.* 2011, 585, 459.
15. Kofod, M. A.; Wampler, D. A.; Pandey, A. S.; Peters, J. W.; Ensign, S. A. Roles of the redox-active disulfide and histidine residues forming a catalytic dyad in reactions catalyzed by 2-ketopropyl coenzyme M oxidoreductase/carboxylase. *J. Bacteriol.* 2011, 193, 4904–4913.
16. Prussia, G. A.; Gauss, G. H.; Mus, F.; Conner, L.; DuBois, J. L.; Peters, J. W. Substitution of a conserved catalytic dyad into 2-KPPC causes loss of carboxylation activity. *FEBS Lett.* 2016, 590, 2991–2996.
17. Vögel, B.; Geyer, K.; Gerlinger, P. D.; Benzstein, S.; Cortina, N. S.; Erb, T. J. Combining promiscuous acyl-CoA oxidase and enoyl-CoA carboxylase/reductases for atypical polyketide extender unit biosynthesis. *Cell Chem. Biol.* 2018, 25, 833–839.
18. Könneke, M.; Schubert, D. M.; Brown, P. C.; Hügerl, M.; Standf fest, S.; Schada von Borzyskowsky, L.; Erb, T. J.;
Stahl, D. A.; Berg, I. A. Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO₂ fixation. Proc. Natl. Acad. Sci. U. S. A. 2014, 111, 8239–8244.

(19) Berg, I. A.; Kockelkorn, D.; Buckel, W.; Fuchs, G. A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in archaea. Science 2007, 318, 1782–1786.

(20) Tcherkez, G. G.; Farquhar, G. D.; Andrews, T. J. Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. Proc. Natl. Acad. Sci. U. S. A. 2006, 103, 7246–7251.

(21) Zhang, L.; Mori, T.; Zheng, Q.; Awakawa, T.; Yan, Y.; Liu, W.; Abe, I. Rational control of polyketide extender units by structure-based engineering of a crotonyl-CoA carboxylase/reductase in antimycin biosynthesis. Angew. Chem. 2015, 127, 13664–13667.

(22) Waterhouse, A.; Bertoni, M.; Bienert, S.; Studer, G.; Tauriello, G.; Gumienny, R.; Heer, F. T.; de Beer, T. A. P.; Rempfer, C.; Bordoli, L.; et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 2018, 46, W296–W303.

(23) Flamholz, A.; Noor, E.; Bar-Even, A.; Milo, R. eQuilibrator—the biochemical thermodynamics calculator. Nucleic Acids Res. 2012, 40, D770–D775.

(24) Vögel, B.; Erb, T. J. Negative’ and ‘positive catalysis’: complementary principles that shape the catalytic landscape of enzymes. Curr. Opin. Chem. Biol. 2018, 47, 94–100.

(25) Rosenthal, R. G.; Vögel, B.; Wagner, T.; Shima, S.; Erb, T. J. A conserved threonine prevents self-intoxication of enoyl-thioester reductases. Nat. Chem. Biol. 2017, 13, 745–749.

(26) Rosenthal, R. G.; Vögel, B.; Wagner, T.; Shima, S.; Erb, T. J. A conserved threonine prevents self-intoxication of enoyl-thioester reductases. Nat. Chem. Biol. 2017, 13, 745–749.

(27) Erb, T. J.; Zarzycki, J. A short history of Rubisco: the rise and fall (?) of Nature’s predominant CO₂ fixing enzyme. Curr. Opin. Biotechnol. 2018, 49, 100–107.

(28) Schada von Borzyskowski, L.; Rosenthal, R. G.; Erb, T. J. Evolutionary history and biotechnological future of carboxylases. J. Biotechnol. 2013, 168, 243–251.

(29) Zarzycki, J.; Brecht, V.; Muller, M.; Fuchs, G. Identifying the missing steps of the autotrophic 3-hydroxypropionate CO₂ fixation cycle in Chloroflexus aurantiacus. Proc. Natl. Acad. Sci. U. S. A. 2009, 106, 21317–21322.

(30) Zarzycki, J.; Fuchs, G. Coassimilation of organic substrates via the autotrophic 3-hydroxypropionate bi-cycle in Chloroflexus aurantiacus. Appl. Environ. Microbiol. 2011, 77, 6181–6188.

(31) Schwander, T.; Schada von Borzyskowski, L.; Burgener, S.; Cortina, N. S.; Erb, T. J. A synthetic pathway for the fixation of carbon dioxide in vitro. Science 2016, 354, 900–904.

(32) Trudeau, D. L.; Edlich-Muth, C.; Zarzycki, J.; Scheffen, M.; Goldsmith, M.; Khersonsky, O.; Avizemer, Z.; Fleishman, S. J.; Cotton, G. A.; Erb, T. J.; et al. Design and in vitro realization of carbon-conserving photorespiration. Proc. Natl. Acad. Sci. U. S. A. 2018, 115, E11455–E11464.