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Synthetic CO_2-fixating biocatalytic cycles: a study on the current strategies and computational optimisation prospects

Bachelor's Thesis

Vlad Andrei Ungureanu – S4728009
BSc. Life Science and Technology, Biomolecular Sciences

Primary supervisor: dr. Maximilian J.L.J. Fürst
Second assessor: Prof. dr. ir. Marco W. Fraaije

Groningen Biomolecular Sciences and Biotechnology Institute (GBB)
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Abstract

The increasing levels of anthropogenic CO_2 emissions in the atmosphere are directly linked to the global warming crisis. This results in a growing scientific interest in harnessing the compound and using it to alleviate the environmental crisis, coupled with the generation of valuable organic products. Biological CO_2 fixation is the most effective mechanism for incorporating the gas in the biosphere at a large scale. However, it does not succeed to outweigh the climatic issue, as a consequence of inefficient biocatalysis and susceptibility to the formation of undesired side products in oxygenic conditions. Therefore, the synthetic biology field proposes new-to-nature biocatalytic cycles meant to surpass these limitations, fixing CO_2 at high rates and generating diverse and useful organic compounds. This thesis aimed to study the current strategies for designing such artificial metabolic networks and identify common motifs, challenges and future prospects. Moreover, it complemented the imminent limitations of these pathways by outlining computational analysis methods such as molecular dynamics simulations, molecular modelling and AI-driven protein engineering endeavours, towards suggesting how CO_2 fixation can be optimised, particularly in regards to carboxylases. All in all, the study suggested how research could improve synthetic CO_2 fixation for a greener future.

Introduction

Global warming represents one of the most distressing crises that humanity is currently facing. Anthropogenic greenhouse gas emissions, primarily CO_2, are predicted to cause an increase of up to 4.5°C in the average global land-air temperature by 2035, as compared to pre-industrial values (McCulloch et al., 2024). This issue calls for interdisciplinary efforts to tackle the accumulation of CO_2 in the atmosphere. Carbon is among Earth's most abundant chemical elements, serving as a fundamental building block for organic compounds including nucleic acids, proteins and lipids. The introduction of carbon and its derivatives into the biosphere occurs naturally through the process of carbon fixation, performed by autotrophic organisms such as plants, most algae, and some bacteria (i.e. cyanobacteria). This way, inorganic carbon is converted into biomass, including organic compounds essential for energy storage and carbon cycling in nature (Santos Correa et al., 2023). The biocatalyst ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is among the most abundant proteins in the biosphere, catalysing the conversion of approximately 90% of inorganic carbon into biomass (Erb and Zarzycki, 2017). The enzyme is primarily associated with the Calvin–Benson–Bassham (CBB) cycle, which assimilates carbon in all plants and algae, as well as in some prokaryotes (Santos Correa et al., 2023). The cycle is part of the photosynthesis process, involving light energy to generate ATP and NADPH, which are essential energy carriers for the assimilation of CO_2. Overall, more than 95% of the 350 Gt of carbon dioxide fixed yearly by autotrophs is funnelled through RuBisCO via

the CBB cycle (McLean et al., 2023). The rate of this process was estimated to be 1-3 nmol min^{-1} mg^{-1} protein (Schwander et al., 2016). Although the mechanism is naturally prevalent, it cannot offset the increasing anthropogenic CO_2 emissions, mainly due to limitations in its biocatalytic machinery. Specifically, RuBisCO captures CO_2 at a rate of 5-10 molecules per second, which is inefficient compared to other enzymes of the carbon metabolism network. Additionally, the protein is prone to forming side products when its substrate, ribulose-1,5-bisphosphate, reacts with atmospheric oxygen (O_2). Studies aimed at improving RuBisCO's activity have found an inverse relationship between its catalytic efficiency and specificity for CO_2 (Erb, 2024). Hence, designing alternative systems for the fixation of CO_2 represents a valuable research frontier to alleviate the environmental effects of the gas emissions.

Towards surpassing the limitations of natural CO_2 assimilation, synthetic biology aims to design and engineer artificial systems with enhanced CO_2 capture and conversion efficiency. This involves creating new-to-nature enzymes and metabolic pathways that enable the generation of novel products and improve capture efficiency beyond the capabilities of natural analogues. To achieve this, however, it is necessary to comprehend the mechanisms of the organic processes and harness them for efficient and stable artificial systems. From a chemical point of view, carboxylation reactions are particularly intriguing when it comes to inorganic carbon fixation, as they enable the integration of C_1 units into target molecules. Carboxylase enzymes (such as RuBisCO) sustain the process, reacting directly with CO_2. Even though atmospheric CO_2 is abundant, its concentration usually does not suffice for carboxylations with the reagent in gaseous phase. Therefore, phase transfer and solubility of CO_2 in solution are crucial parameters for carboxylation chemistry. Due to the hydration of CO_2 in solution, its availability is pH-dependent, marking bicarbonate (HCO_3^-) as its most prominent derivative at neutral/alkaline pH values. Moreover, for the carbon assimilation reaction to occur, a reductive agent such as NADPH is required due to the high oxidation state of carbon in CO_2. The carboxylation reaction follows a general mechanism with high similarity across most carboxylases (Figure 1). Initially, the substrate is converted into a nucleophile (enol or enolate). The compound is then stabilised by cofactors (such as metal ions) or enzyme residues. Subsequently, the carboxylase enzyme accommodates and activates the CO_2 molecule, facilitating the nucleophilic attack of the substrate on the inorganic carbon. Follow-up reactions, including reductions, eliminations, or cleavages, complete the process (Bierbaumer et al., 2023). Engineering carboxylases to optimise CO_2 capture rates represents a significant direction for the development of efficient synthetic systems for inorganic carbon fixation. Grasping the principles, challenges and opportunities of synthetic CO_2-fixing mechanisms is crucial. In light of these considerations, this review assessed the current strategies in artificial CO_2 fixation, as outlined in recent research. Consequently, the first research sub-question we addressed is identifying common motifs involved in the development of synthetic CO_2 fixation systems.

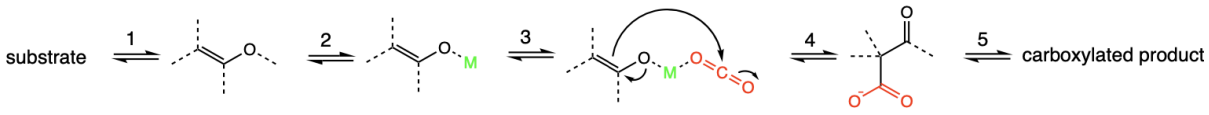


Figure 1 | The steps of CO_2 fixation via carboxylation. The pathway involves the steps of enol formation **(1)**, enol stabilisation **(2)** via cofactors/residues (M), CO_2 activation **(3)**, C-C bonding **(4)** and follow-up reactions **(5)**. The image was generated in ChemDraw, based on a figure of Bierbaumer et al., 2023.

The field of enzyme engineering stands out as a means for the optimisation of biocatalysts, aiming to improve their performance, stability, and substrate specificity (either enhancing it or promoting promiscuity). Protein optimisation can be performed via directed evolution, which subjects genes to non-targeted iterative rounds of mutagenesis, followed by selecting mutants with desired properties from screening assays. This process mimics natural selection. In contrast, rational protein design involves targeted protein alterations through point mutations or, alternatively, via the de novo design of domains and motifs (Vidal et al., 2023). While directed evolution can be highly effective, it can also be costly, labour-intensive, and time-consuming. Computational biology brings up a complementary, theoretical, approach to enzyme engineering by predicting relevant mutations to develop desired variants (Planas-Iglesias et al., 2021). Additionally, related techniques can be used for de novo development of proteins. In silico enzyme development can accelerate the engineering goals significantly, optimising processes and reducing costs. The process mainly relies on obtaining atomic structures for the proteins of interest and identifying amino acid sequences which can improve set functions. Energy-related parameters of novel proteins (i.e. electrostatic interactions, backbone torsions, disulfide bond energies) are critical in such experiments and must be considered (Meinen and Bahl, 2021). Computational biology extends beyond the design and engineering of enzymes. The development of machine learning (ML) algorithms is a greatly relevant milestone in this context, ensuring predictions based on past experimental results. AlphaFold, for example, predicts protein structures using data from past research in fields like genomics, bioinformatics and biophysics for training its algorithm. Although such methods lead to a better structural characterisation of proteins by proposing various models, predicting the catalytic functions of proteins remains a striking challenge for computational biologists (Korbeld and Fürst, 2023). Despite the ongoing development and various challenges, computational biology plays a crucial role in protein design, modelling and engineering. Computational methods have the potential of optimising artificial CO_2 fixation systems and the corresponding biocatalysts. Since these techniques are new and under constant development, there is still a gap in understanding what these optimisation prospects could generally look like for artificial CO_2 fixation. Thus, we brought up a second research sub-question, assessing how computational methods can complement in vitro protein studies, particularly in the case of CO_2-fixing enzymes.

The accretion of atmospheric CO_2 leads to global warming, one of the most prominent crises faced by humanity. Systems of artificial fixation and harnessing of the gas for the synthesis of organic products are currently being developed as alternatives to the inefficient autotrophic analogues. Considering the recent advances in computational protein characterisation and design, this thesis aimed to showcase how computational biology can improve synthetic CO_2 fixation cycles. This way, we set out to bridge the knowledge gap regarding the integration of computational methods for optimising synthetic CO_2 fixation. The approach followed here involved assessing current in vitro/in vivo synthetic biology strategies, emphasising the role and diversity of carboxylases due to their imminent role in fixing C_1 units. Moreover, we described how synthetic pathways can be integrated in order to complement natural mechanisms effectively. The thesis further explored the potential of computational methods to offset the limitations of the enzymes involved in synthetic CO_2 fixation and improve the understanding of their functionality. By assessing such methods in the context of optimising carboxylases and synthetic CO_2 fixing systems, we posed the question: how do targeted approaches of computational protein development contribute to the improvement of synthetic CO_2 fixation biocatalytic cycles?

Synthetic Biology Strategies for CO_2 Fixation

In biological systems, the fixation of CO_2 is regulated by the physiological needs of the cell for optimal growth. Designing in vitro biocatalytic systems alleviates these limitations, enabling the development of efficient and specific pathways for converting CO_2 into useful organic compounds (Luo et al., 2022). Cell-free studies facilitate the eventual application of these systems in vivo, by gradually optimising CO_2-fixing pathways independently from the cellular environment. Synthetic systems often lack the dynamic functionality of natural pathways, which can adapt relative to the biosynthetic needs of the cell (Diehl et al., 2022). Therefore, developing new-to-nature pathway systems in vivo is among the significant challenges currently faced by synthetic biology researchers. The scale of the challenge increases with the intricacy of the designed pathway, due to the risk of creating new, undesired, side reactions between the enzymatic components of the artificial systems and the native cellular networks of the cell (Luo et al., 2023). While natural mechanisms have been transferred into the cellular machinery of *Escherichia coli (E. coli)* previously, designing new-to-nature pathways of CO_2 fixation that can function inside cells is a great opportunity to broaden the scope of their applicability (Gleizer et al., 2019). Moreover, in vivo systems can complement the autotrophic and cell-free pathways by raising the rate of CO_2 fixation in nature at a large scale, and enabling proactive comparisons between the systems. Thus, this review claims that the design of in vivo CO_2-fixing systems is a critical research frontier towards the development of efficient large scale systems of outweighing CO_2 emissions. We further look at three synthetic systems described in recent research with distinct features, enabling a qualitative overview of the current strategies of artificial CO_2 fixation.

The self-replenishing in vitro rGPS-MCG pathway

Considering that most natural CO_2 fixation pathways are organised in self-replenishing cycles, having reaction products as intermediates, Luo et al. proposed a system for the synthetic assimilation of CO_2. The authors highlighted two advantages of this biocatalytic configuration. First, the fact that all the intermediates of a cycle are reaction products makes it possible to ensure a large variety of useful organic compounds to be withdrawn from the system, provided that they are not drained beyond replenishment (for example, the CBB cycle outputs C_3 to C_7 products). Second, the self-replenishing character of these systems renders them autocatalytic, raising the cycle rates as per the accumulation of intermediate products (Luo et al., 2022). Keeping this in mind, the authors proposed an oxygen-insensitive, self-replenishing CO_2 fixation system: the rGPS-MCG cycle (Figure 2). This cycle was able to convert C_1 compounds (CO_2 and HCO_3^-) to useful organic products including pyruvate and acetyl-coenzyme A (acetyl-CoA). Further, it did so with a relatively low energetic input of 5 ATP molecules per 2 bicarbonate molecules, as opposed to the 9 ATP molecules used by the CBB cycle. It was also able to sustain a steady state for about 6 hours (Luo et al., 2023; Schwander et al., 2016). These characteristics stood out, outlining the quality and feasibility of the rGPS-MCG cycle.

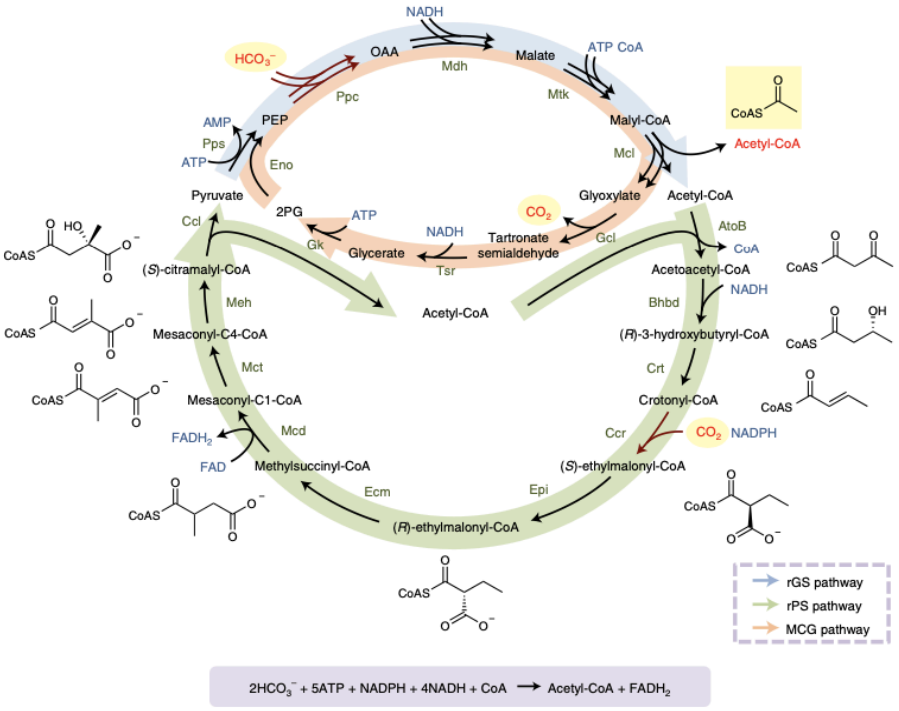


Figure 2 | Overview of the rGPS-MCG system. The cycle consists of the rGS pathway (blue), the rPS pathway (green) and the MCG pathway (orange). The latter is composed of the rGS and glycerate pathways. Overall, the cycle fixes 2 molecules of bicarbonate to produce one molecule of acetyl-CoA. The carboxylation steps are catalysed by the enzymes Ppc and Ccr (highlighted in red). The figure was adapted from Luo et al., 2022.

The system's design commenced with the phosphoenolpyruvate (PEP) carboxylase (Ppc), which catalyses the conversion of PEP (C_3) to the C_4 oxaloacetate (OAA) molecule. The enzyme lacks oxygenase activity, rendering it unsusceptible for reactions with atmospheric oxygen, but has a high affinity towards the bicarbonate it fixes. For OAA to be converted back to PEP, the loss of one carbon unit was required. Thus, the study introduced the reductive glyoxylate synthesis (rGS) pathway, which fixes one molecule of bicarbonate and then converts pyruvate (the precursor of PEP) to glyoxylate and acetyl-CoA (C_2 compounds). The usage of a pyruvate carboxylase (Pyc) to convert pyruvate to OAA directly was tested but found to be less efficient than the step-wise conversion described above. The cycle was completed by the glycerate pathway, which converts glyoxylate to pyruvate via glycerate, by losing a molecule of CO_2. The systems are connected as the malyl-CoA-glycerate (MCG) pathway.

For the self-replenishing cycle to be complete, the C_2 acetyl-CoA was converted to the C_3 pyruvate. Naturally, the pyruvate-ferredoxin oxidoreductase (PFOR) of the Wood-Lindahl pathway fixes CO_2 to acetyl-CoA to generate pyruvate. Considering the oxygen sensitivity of PFOR and the limitations of other pathways in performing this step, a functionally equivalent system was created with the crotonyl-CoA carboxylase/reductase (Ccr) enzyme. The choice of this enzyme was based on its central role in the previously reported crotonyl-CoA-ethylmalonyl-CoA-hydroxybutyryl-CoA (CETCH) synthetic cycle. In the case of the system described here, Ccr performs the reductive carboxylation of crotonyl-CoA (Figure 3) to (S)-ethylmalnoyl-CoA, using NADPH as a reducing equivalent (Erb et al., 2007). Overall, the enzyme has superior catalytic activity and is oxygen insensitive, similar to the Ppc carboxylase. Therefore, the conversion of acetyl-CoA to pyruvate was proposed by Luo et al. through the reductive pyruvate synthesis (rPS) pathway. This pathway uses two acetyl-CoA molecules and fixates CO_2, forming the mesaconyl-C1-CoA intermediate and splitting its successor, (S)-citramalyl-CoA, in pyruvate and acetyl-CoA. Overall, the rPS pathway designed by Luo et al. is responsible for the conversion of acetyl-CoA to pyruvate by fixing one CO_2 molecule and is coupled to the rGS pathway which transforms the pyruvate to acetyl-CoA and glyoxylate. Interestingly, the systems have also been proposed by computational studies before the experimental work described here (Löwe and Kremling, 2021; Bar-Even et al., 2010).

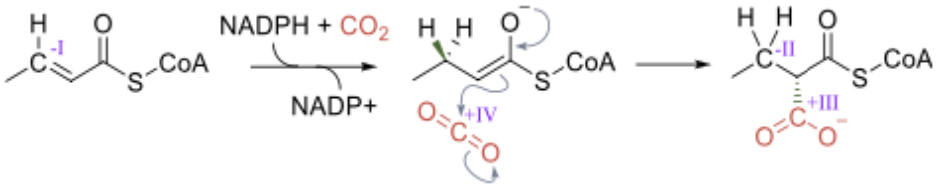


Figure 3 | The catalytic cycle of the crotonyl-CoA carboxylase/reductase enzyme. This figure was adapted from Bierbaumer et al., 2023.

The reductive glyoxylate-pyruvate synthesis (rGPS) cycle was complemented by the MCG pathway, forming a network that produces a range of useful organic products directly from CO_2 and its derivatives: acetyl-CoA (C_2), pyruvate (C_3) and malate (C_4). The demonstration of the rGPS-MCG pathway's success was performed via ^{13}C-labelling of the bicarbonate and formate (converted to CO_2 with formate dehydrogenase) fed into the system, enabling the detection of labelled products. The cellular balance of cofactors is regulated by various mechanisms, which were substituted by the authors with optic-fibre systems of automatic real-time detection of the cofactors (Luo et al., 2022). Thus, the self-replenished pathway maintained a steady operation mode for 6 hours, reaching a CO_2 fixation rate of 28.5 nmol min^{-1} mg^{-1} core protein. This value renders the synthetic cycle more efficient than the CBB cycle. The inactivation of the biocatalysts turned out to be the main limitation of the system. While enzymes like Ppc were most likely destabilised by protein oxidation, Ccr showed an intrinsically unstable character in the ambient conditions of the experiment. Because of this, the authors encouraged the finding of more stable enzymes or the further optimisation of Ccr via directed evolution and complementary rational design approaches (Luo et al., 2022).

The nature-inspired in vitro HOPAC pathway

The 3-hydroxypropionate (3HP) bi-cycle (Figure 4.A) converts acetyl-CoA to pyruvate from three molecules of bicarbonate, fixed in two separate rounds. The pathway utilises 13 enzymes catalysing 19 conversion steps to organic compounds like glyoxylate (Zarzycki and Fuchs, 2011). To enhance this system, the hydroxypropionyl-CoA/acrylic-CoA (HOPAC) cycle was designed (Figure 4.B) as a more energetically feasible alternative (2 ATP input) with a similar topology to the 3HP mechanism of *Chloroflexus aurantiacus* green nonsulfur bacteria (McLean et al., 2023).

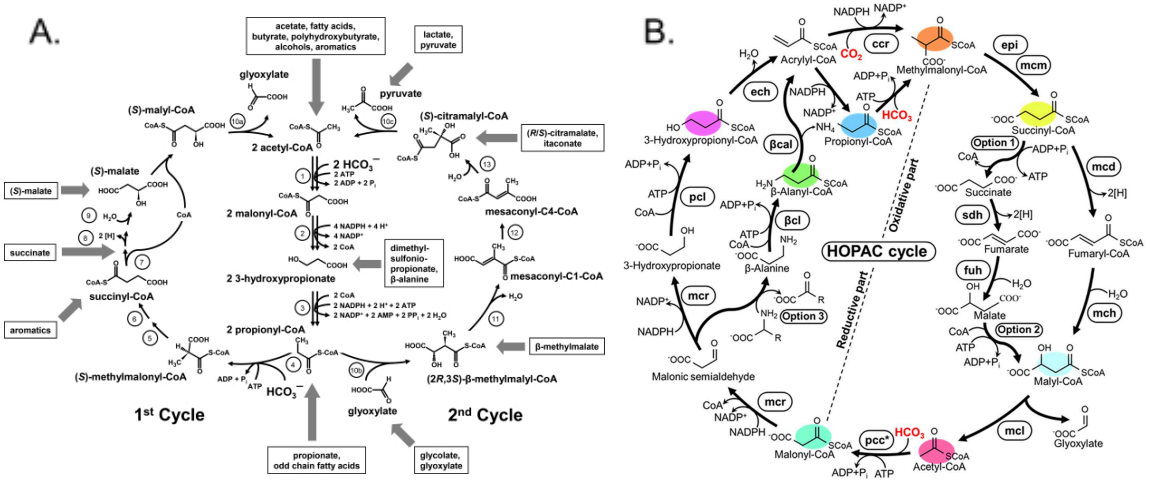


Figure 4 | Overview of the nature-inspired HOPAC cycle. The figure depicts the autotrophic 3HP bi-cycle of *Chloroflexus aurantiacus* **(A)** and the synthetic pathway designed with the natural analogue as a base **(B)**. The HOPAC cycle consists of a reductive part and an oxidative part, producing glyoxylate from bicarbonate and CO_2. The cycle's reaction is: **$2CO_2$** + 3NADPH + **2ATP** + FAD → **glyoxylate** + $3NADP^+$ + H^+ + 2ADP + $2P_i$ + $FADH_2$. The panels of the figure were adapted from Zarzycki and Fuchs, 2011, and McLean et al., 2023.

Functionally, the cycle was divided into two pathways based on the overall biochemical reactions they enclose (reductive and oxidative). The system commences with the assimilation of bicarbonate onto acetyl-CoA via the ATP-dependent propionyl-CoA carboxylase (Pcc), yielding malonyl-CoA which can be further converted into the compound 3-hydroxypropionate-CoA (3HP). Subsequently, the reductive pathway finishes with the generation of acrylic-CoA and a second fixation step in which the Ccr carboxylase assimilates CO_2, generating (2S)-methylmalonyl-CoA. Next, the C_4 compound is converted to succinyl-CoA and, in the next step, malyl-CoA. At last, the malyl-CoA is cleaved into the compound initiating the cycle, acetyl-CoA, and glyoxylate which is the primary output of the HOPAC pathway. In order to drive the reactions forward, glyoxylate was further reduced to glycolate (McLean et al., 2023).

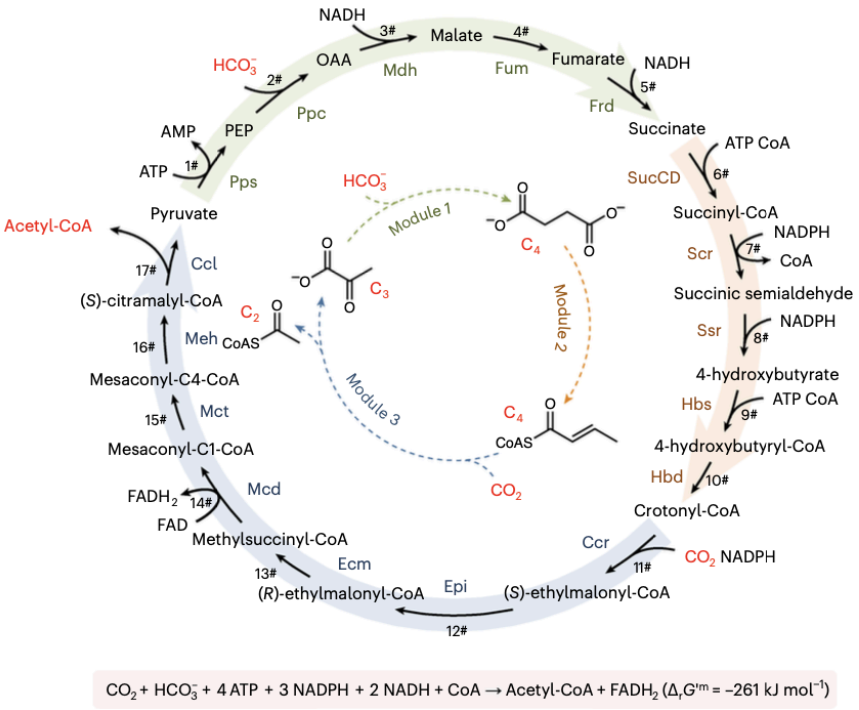
The authors of the study highlighted alternative routes for both pathways of the HOPAC cycle. First, they introduced the β-alanine route of the reductive pathway. Here, the malonic semialdehyde (successor of malonyl-CoA) was converted to the β-alanine compound which was further bound to coenzyme A (CoA), deaminated and carboxylated towards obtaining (2S)-methylmalonyl-CoA. A different approach to the Ccr-catalysed carboxylation reaction was also presented in the study, employing a step-wise conversion of acrylic-CoA to propionyl-CoA and, eventually, (2S)-methylmalonyl-CoA. This was achieved using the propionyl-CoA synthase (Pcs) enzyme, coupled with the Ppc carboxylase. Interestingly, Pcs forms a multicatalytic system which can also drive the dehydration of 3HP to acrylic-CoA (McLean et al., 2023). Past studies engineered the enzyme to induce carboxylation towards acrylic-CoA (Bernhardsgrütter et al., 2019). For the oxidative pathway of the HOPAC cycle, an alternative course of turning succinyl-CoA to (S)-malyl-CoA was proposed: the “free acid route”. Here, succinyl and CoA were cleaved, and the former was oxidised to fumarate, hydrated to malate and bound back with coenzyme A.

The reductive carboxylation reaction catalysed by Ccr renders the cycle more energy efficient than its natural counterpart, using one less ATP molecule. The Ccr-based variant of the HOPAC network was further improved by coupling the reactions to electron transport proteins and other cofactors, generating ~350 µM glycolate from 200 µM acetyl-CoA ($HOPAC_{Ccr}$ 3.0). The Pcs/Pcc-driven alternative to the Ccr-catalysed carboxylation was proven feasible and well-functional too, but could not outperform $HOPAC_{Ccr}$ 1.0. Because of this, the ML-based METIS workflow was chosen to optimise the Ccr-based cycle. This active learning system was trained on sets of results to suggest novel approaches to raising the efficiency of the cycle. It did so by searching the combinatorial space of the in vitro system in iterative cycles in order to find optimal strategies (Pandi et al., 2022; McLean et al., 2023). Hence, following 8 rounds of METIS-guided optimisation, a production of ~1500 µM glycolate (from 200 µM acetyl-CoA) was reached by $HOPAC_{Ccr}$ 4.0.

The HOPAC cycle is a biocatalytic system utilising a total of 11 enzymes from 6 different organisms, converting acetyl-CoA to glyoxylate. The CO_2 fixation rate of the HOPAC cycle is 2.4 nmol CO_2 min^{-1} mg^{-1} protein, rendering it comparable to synthetic analogues such as the rGPS-MCG cycle described above. The authors suggest that the HOPAC cycle could be viable for in vivo applications, highlighting the promising potential of the Pcs/Pcc-based alternative to carboxylations driven by the Ccr enzyme. Pcs, most specifically, was predicted to fulfil multi-catalyzing roles and reduce the ATP cost of the HOPAC cycle, provided that protein engineering efforts are sustained for its development.

The modular THETA pathway with in vivo applications

One of the open challenges of synthetic CO_2 fixation remains the design of novel in vivo viable pathways. Using rational protein design and ML-guided optimisation methods, the reductive tri-carboxylic acid branch/4-hydroxybutyryl-CoA/ethylmalonyl-CoA/acetyl-CoA (THETA) synthetic cycle (Figure 5) was developed for the conversion of CO_2 to acetyl-CoA, at the cost of 4 ATP molecules per fixed CO_2 (Luo et al., 2023). The design of the THETA cycle revolved around the use of naturally existing carboxylases with favourable catalytic properties, surpassing RuBisCO by more than an order of magnitude: Ppc and Ccr. The study deems these proteins as the fastest CO_2-fixing enzymes studied up until the point of its publication, as further dwelt upon in the following section of this thesis.



$CO_2 + HCO_3^- + 4\ ATP + 3\ NADPH + 2\ NADH + CoA \rightarrow Acetyl\text{-}CoA + FADH_2\ (\Delta_r G'^m = -261\ kJ\ mol^{-1})$

Figure 5 | Overview of the THETA cycle. The cycle consists of three modules, shown in green, orange and blue. Overall, the cycle fixes CO_2 to produce acetyl-CoA, using the Ppc and Ccr enzymes for carboxylation. This figure was adapted from Luo et al., 2023.

Towards designing a cycle enclosing reactions catalysed by Ppc and Ccr, the authors proposed a modular approach to the THETA pathway, splitting it into three segments. Based on the native activity of the carboxylase, module 1 was built to convert pyruvate to succinate via PEP synthesis, Ppc-driven bicarbonate assimilation and three reductive steps of the CBB cycle. The Ccr-catalysed conversion of crotonyl-CoA to (2S)-ehtylmalonyl-CoA via CO_2 fixation was joined in module 2 by three reactions from the 3HP bi-cycle, yielding acetyl-CoA and pyruvate (module 1's starting compounds) as products. The carboxylation blocks were linked by module 2, enclosing 4 reactions from other naturally occurring cycles to convert succinate into crotonyl-CoA (Luo et al., 2023). The THETA cycle has been identified previously as the reductive citramalyl-CoA pathway, via a computational study without experimental support (Löwe and Kremling, 2021).

Commencing the cycle from 200 µM of distinct intermediates (either pyruvate, fumarate or succinate) made it possible for the authors to conclude that the fumarate reductase enzyme was a limiting factor for the in vitro application of the THETA cycle, due to its redox activity. This was proven by the LC-MS-based supervision of the CoA esters, displaying a 3.7-fold increase in acetyl-CoA concentration when succinate was chosen as the starting compound, rather than pyruvate. THETA 3.0 was generated following the implementation of a bypass that offset this limitation, yielding a higher production (~200 µM acetyl-CoA in 60 minutes) than THETA 1.0 (initial). The CO_2-fixation rate of the system was found to be 2.7 nmol min^{-1} mg^{-1} protein (Luo et al., 2023). The THETA cycle's potential was further explored using the METIS workflow. The THETA 3.9.9 cycle was, thus, obtained, yielding 1,150 µM acetyl-CoA from 200 µM pyruvate (~5-fold increase relative to THETA 3.0).

We further highlight the defining feature of Luo et al.'s study, the integration of the THETA cycle into the native metabolic network of bacterial *E. coli* cells (Figure 6). The modules were introduced individually in the auxotrophic variants of the bacterium. In strain JCL301, the CBB cycle was disconnected from glycolysis via the deletion of genes responsible for the natural conversion of pyruvate to acetyl-CoA (*aceEF*, *poxB* and *pflB*). Moreover, the glyoxylate shunt of the CBB cycle (isocitrate transformation to malate) was severed by deleting the *sucAB* and *aceA* genes. This process rendered the strain dependent on external feeding with acetate. The auxotrophic cells could not produce succinate naturally, which affected the synthesis of methionine and lysine amino acids. Succinate could, thus, only be produced by the auxotrophic *E. coli* cells via module 1 (Figure 6.A), resulting in cell growth. Similarly, module 2 was successfully introduced to strain SL2, a JCL301 variant with two additional deletions (*Δkbl* and *ΔltaE*) to disturb the synthesis of acetyl-CoA from threonine. Acetyl-CoA is critical for cell growth and was synthesised in vivo from the crotonyl-CoA product of module 2, via an additional biocatalytic mechanism involving three conversion steps (Figure 6.B). The HH61 *E. coli* strain was obtained by integrating five new-to-the-cell genes (*epi*, *ecm*, *mct*, *meh* and *ccl*) into the acetyl-CoA auxotroph SL2.

Module 3 was, thus, implemented in the HH61 cells (Figure 6.C). Due to the accumulation of undesired side products, a bypass was designed to utilise these compounds (mesaconate and methylsuccinate) to synthesise pyruvate. The results of the in vivo implementation of the cycle were validated via ^{13}C-labelling experiments (Luo et al., 2023).

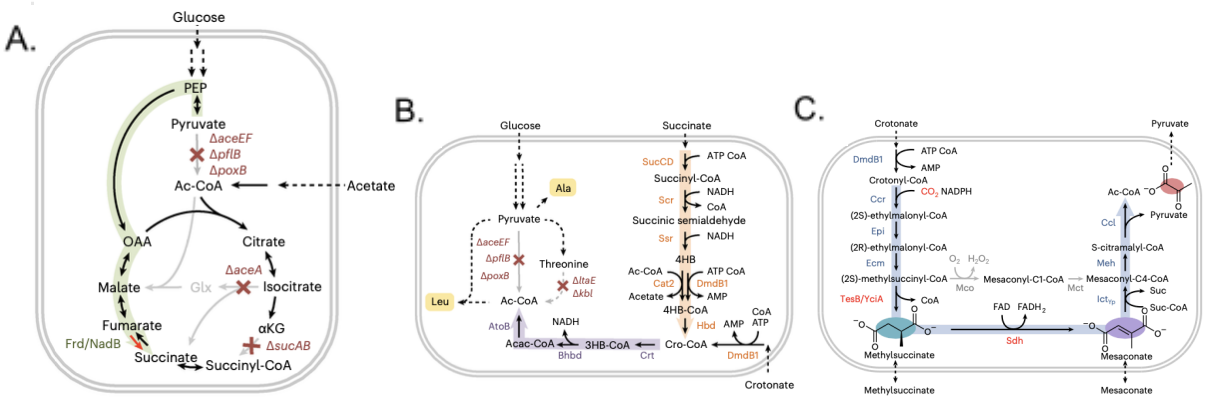


Figure 6 | The modular implementation of the THETA cycle in vivo. The figure presents the three THETA cycle modules and their integration into auxotrophic *E. coli* cells. For module 1, the JC301 strain was developed to rely on the artificial cycle for succinate production, following the deletion of genes for the downstream steps of glycolysis **(A)**. Module 2 was implemented in SL2 auxotrophs, lacking the genes for natural acetyl-CoA production. Crotonyl-CoA is converted to acetyl-CoA via an additional biocatalytic pathway, marked in purple **(B)**. Lastly, module 3 was applied in HH61 cells, producing pyruvate and acetyl-CoA after the introduction of five enzymes new to the genetic network of *E. coli* **(C)**. All three panels were adapted from Luo et al., 2023.

The THETA cycle was successful in converting CO_2 to pyruvate, with a carbon fixation rate that is comparable to the analogues that we described in this thesis (Table 1). Moreso, the cycle was integrated into an in vivo network, proving the possibility of using synthetic biology for such purposes. The authors of the study further encouraged additional efforts of rational protein engineering for optimising the pathway even more, to increase the chances of bringing this system of orthogonal enzymes to life effectively (Luo et al., 2023).

Table 1 | Comparison of synthetic CO_2-fixing cycles with the CBB cycle. The table displays values for the parameters of interest (CO_2-fixation rate, cofactor requirements and products) corresponding to the rGPS-MCG, HOPAC and THETA pathways.

Cycle	CO_2-fixation Rate (nmol min^{-1} mg^{-1} protein)	Cofactor Requirements		Cycle Product	Reference
		ATP	NAD(P)H		
rGPS-MCG	28.5	5	5	Acetyl-CoA	Luo et al., 2022
HOPAC	2.4	2	3	Glyoxylate	McLean et al., 2023
THETA	2.7	4	5	Acetyl-CoA	Luo et al., 2023
CBB	1-3	9	6	Glyceraldehyde-3-phosphate	Schwander et al., 2016

Characterising the most efficient CO_2-fixing enzymes structurally and functionally

The PEP carboxylase and crotonyl-CoA carboxylase/reductase enzymes hold prominent roles in the synthetic CO_2-assimilating pathways described above. Multiple studies report that the enzymes are fast, O_2 resistant and drive thermodynamically favourable reactions (Luo et al., 2023; Recabarren et al., 2023). Hence, this thesis looks briefly into the specifics of these enzymes' structural and functional characteristics.

The PEP carboxylase

As explicitly described in the presentation of the rGPS-MCG synthetic CO_2-fixing pathway, the phosphoenolpyruvate carboxylase catalyses the formation of OAA from PEP, fixing one molecule of bicarbonate in the process (Luo et al., 2022). Similarly to the RuBisCO enzyme, Ppc uses Mg^{2+} ions as cofactors in its active site. It is coordinated by four water molecules and the residues glutamate and aspartate (Figure 7.A). During the catalytic reaction, Ppc accommodates intermediates like pyruvate enolate anions which are stabilised by the metal ions. As Ppc keeps CO_2 in a hydrophobic pocket in close proximity to the reactive enolate species, side reactions with oxygen and protons (from the water molecules) are suppressed during the catalytic steps of the enzyme (Kai et al., 2003; Bierbaumer et al., 2023).

The crotonyl-CoA carboxylase/reductase

The Ccr enzyme is the best-studied enoyl-CoA carboxylase/reductase (ECR). Naturally, this enzyme family does not catalyse CO_2 fixation but rather assimilates carbon from acetate, using NADPH as a cofactor molecule (Figure 7.B) in the ethylmalonyl-CoA pathway (Erb et al., 2007; Bierbaumer et al., 2023). The ECR enzymes catalyse the reductive carboxylation of α and β-unsaturated acyl-CoA thioesters, like crotonyl-CoA. Ccr-driven catalysis is dependent on four conserved residues of the protein: Phe170 (water shielding), Asn81 (CO_2 stabilisation), Glu171 and His365. The last two amino acids are responsible for maintaining a water molecule bridge, preserving a hydrogen bond network in the active site (Recabarren et al., 2023). The Ccr enzyme surpasses Ppc in terms of efficiency and catalytic activity, as it is considered to be the fastest-known natural carboxylase (Schwander et al., 2016). In the absence of CO_2, Ccr catalyses the formation of the "C_2-adduct" - a species speculated to be involved in storing high-energy enolates in conditions lacking the electrophilic carbon source. By reducing the conversion rates of the activated enolate back to the substrate, this compound is thought to raise the efficiency of carboxylations catalysed by Ccr (Stoffel et al., 2019). The following section of this review commences with an in-depth exploration of the C_2-adduct and explains how integrated computational methods were able to reveal its significance in CO_2 fixation.

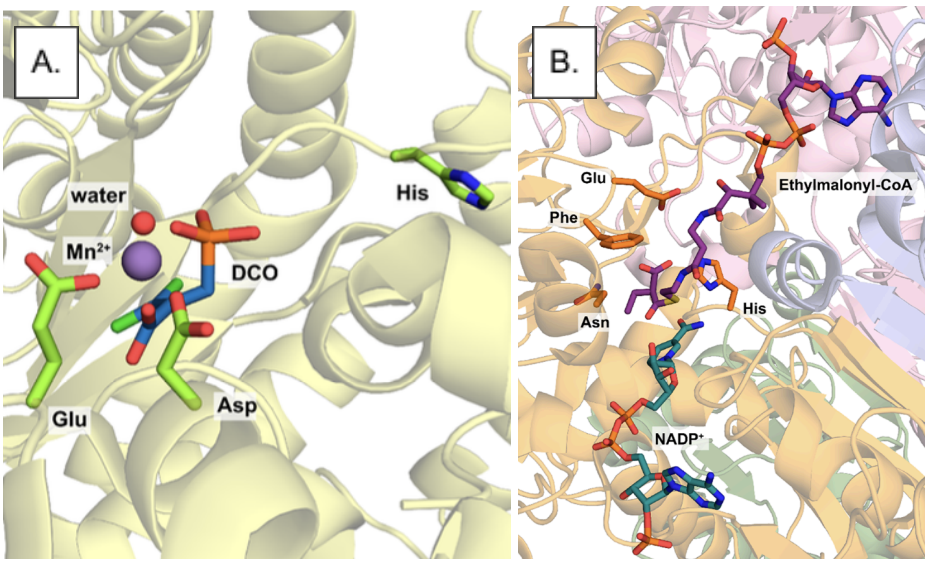


Figure 7 | Overview of the active sites of Ppc and Ccr. The first panel of this figure presents the active site of the *E. coli* Ppc enzyme (PDB 1JQN) when bound to Mn^{2+} (instead of the natural cofactor Mg^{2+}) and a substrate analogue, DCO **(A)**. The second panel reveals the active site of *Kitasatospora setae* Ccr (PDB 6OWE) when bound to the ethylmalonyl-CoA product of carboxylation. The panel also shows the $NADP^+$ cofactor and the key residues of the active site: Phe, Asn, Glu and His **(B)**. The panels were adapted from Bierbaumer et al., 2023.

Harnessing Computational Biology for CO_2 Fixation Enhancement

The field of computational biology is on the rise. Even though many technologies are still under development and training, many computational (particularly ML-based) methods are part of current research approaches. We highlighted this previously, and explained how prediction-based algorithms were able to design synthetic systems like THETA and rGPS-MCG before their experimental application. Moreso, the METIS workflow turned out to be a viable choice to improve enzymes, being applied in the CO_2-fixing cycles described above (Luo et al., 2022; McLean et al., 2023; Luo et al., 2023). We further looked into the employment of computational studies in the context of CO_2 fixation, commencing from the discussion of the C_2-adduct and diverging towards future directions of interest.

The formation of C_2-adducts, studied with molecular dynamics (MD) simulations

While the roles and potential of ECR enzymes (especially Ccr) are well-known, knowledge of their underlying efficiency-ensuring mechanisms is missing. Getting to understand the way these mechanisms are built deepens knowledge of CO_2-fixing enzymes and brings up many possibilities to engineer novel biocatalysts for efficient and fast synthetic pathways of inorganic carbon assimilation. The identification of a C_2-adduct, formed via the addition of the NADPH cofactor to the crotonyl-CoA substrate (Figure 8), has been a key breakthrough towards understanding the mechanisms of Ccr-driven carboxylations (Vögeli et al., 2018). The compound was primarily found in reduction reactions lacking CO_2. However, it was also

associated with an alternative pathway of carboxylation (Rosenthal et al., 2014). Adduct formation between substrates and NAD(P)H nicotinamide rings is not uncommon, holding inhibitory roles towards enzymes in most cases. Since the C_2-adduct compound of Ccr could be identified by Recabarren et al. during carboxylation reactions, its role was assumed not to be of inhibitory nature. The authors of the study assessed the reaction mechanisms of the *Kitasatospora setae* Ccr, highlighting the two mechanisms of carboxylation that the enzyme catalyses: the direct one and the C_2-adduct mechanism. These reaction pathways were further studied with molecular dynamics (MD) simulations, computational tools for predicting the movement of atoms/molecules and the corresponding response to different scenarios. The authors coupled the MD simulations with quantum mechanics to reveal the reactions' energetic backgrounds (Recabarren et al., 2023; Hollingsworth and Dror, 2018).

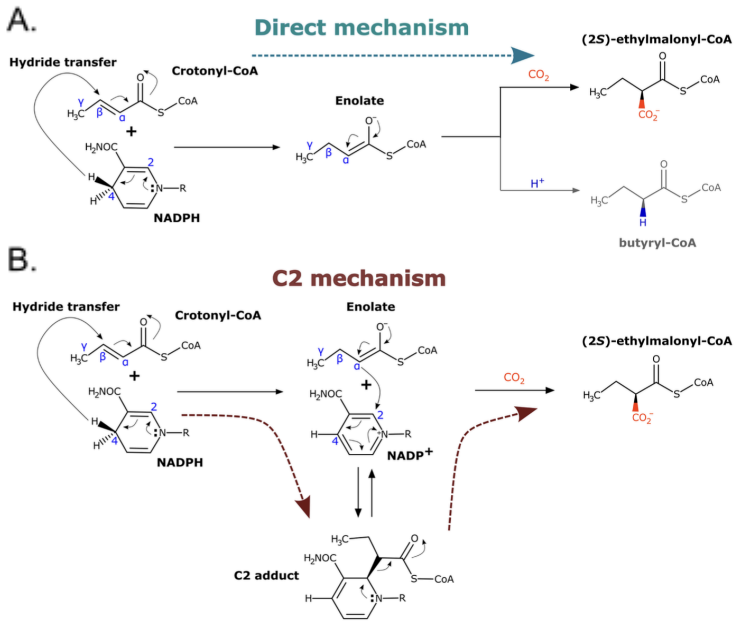


Figure 8 | The two possible carboxylation routes catalysed by Ccr. The figure shows the direct **(A)** and C_2 adduct **(B)** reaction mechanisms catalysed by the Ccr enzyme. The latter involves the formation of a C_2 adduct which is further carboxylated. The figure was adapted from Recabarren et al., 2023.

By studying the direct mechanism of Ccr-driven carboxylation with MD simulations, it was discovered by Recabarren et al. that the hydride transfer is the limiting step of the process, with a free energy of 23.2 kcal/mol. Similar analyses revealed that the formation of the adduct from the enolate intermediate is thermodynamically and kinetically favourable (free energy of 1.9 kcal/mol), showing that the compound is part of Ccr's enzymatic product network. It also clarifies why the C_2-adduct forms both in the presence and absence of CO_2. Lastly, the authors showed that the adduct is more stable compared to the enolate. This suggests that it represents an efficient way of "storing" the reactive molecule. By doing so, the backwards reaction towards the substrate is not favoured but carboxylation is. Besides supporting the findings of Rosenthal et al., the study proved how computational MD can improve the understanding of CO_2 fixation mechanisms.

Conformational dynamics of Ccr studied with computational models and simulations

Understanding the efficiency and reaction mechanisms of enzymes is complex. Gomez et al. reveal that the oligomeric crotonyl-CoA carboxylase/reductase enzyme is a dimer of dimers, each unit displaying open/closed configurations. Thus, their research set out to assess the interactions between Ccr substrates/CO_2 with the carboxylase in its open state, before its change to a closed conformation. Bearing this in mind, the authors hypothesised that the conformational changes of the Ccr enzyme, after it binds the substrate, have a role in the affinity of the biocatalyst for CO_2 by promoting its fixation. MD simulations were carried out using the Ccr tetramer, towards studying its conformational changes and interactions with CO_2. These simulations computed the chances of finding CO_2 next to Ccr's active site.

The results of the study supported the hypothesis described above, revealing that CO_2 binds preferentially onto the open active state of Ccr and resides for a longer time when the substrate is bound. Based on the results of the MD simulations, the authors outlined a conformation-dependent mechanism of CO_2 fixation via Ccr, showing changes across the states of the dimers as they interacted with cofactors during the reactions. This way, the research was able to bridge one of the knowledge gaps regarding Ccr's activity and reaction mechanisms, paving the way for better new-to-nature synthetic pathways and enzymes (Gomez et al., 2023). Here, we suggest that by grasping the interaction dynamics of substrates and enzymes, it is possible to identify biocatalytic motifs causing specificity in these interactions. By identifying these sites and consequently elevating their functions, we believe that synthetic CO_2 fixation systems can be improved.

Protein engineering using ML-based methods

Understanding the reaction mechanisms and functions of enzymes makes it possible to design novel enhanced proteins for CO_2 fixation. The Glycolyl-CoA carboxylase (Gcc) is a new-to-nature biocatalyst, designed through structure-guided approaches and screening of mutagenesis libraries of the Pcc enzyme from *Methylorubrum extorquens*. Gcc is one of the key enzymes of the synthetic tartronyl-CoA (TaCo) pathway and is responsible for catalysing the carboxylation of glycolyl-CoA (Marchal et al., 2023). To harness the full potential of the protein, the study of Marchal et al. employed an ML-based algorithm as a means to filter variants with single mutations from a Gcc mutagenesis library, reducing the efforts of experimental screenings in the search for an ideal enzymatic candidate.

From a dataset of 3000 lysate-based enzyme screens, a subset of 161 was deemed to be representative and relevant for the training of the algorithm, with sequences covering a wide range of biocatalytic activity parameters. All single mutations of the Gcc enzyme were, then, generated and sorted through the algorithm by a confidence criterion calculated by

the model. From this ranked list of predicted catalytic efficiencies, a total of 10 variants were eventually picked for in vitro biochemical assessment, out of which only 9 were active. In the end, the authors found two Gcc mutant variants with beneficial performance: G20R and L100N. To understand the origins of the catalytic improvement shown by these enzymes, the corresponding cryo-EM structures were solved at resolutions of 2.05 Å and, respectively, 2.31 Å. In the G20R Gcc variant, the position of the arginine on a flexible loop turned out to be involved in the stabilisation of the enzyme and the positioning of CoA molecules efficiently. The asparagine L100N residue was found to be near the rotameric His143, which has roles in substrate binding in the active site of Gcc. By influencing the slight movement of this rotamer, the asparagine was assumed to improve catalytic activity for the enzyme (Marchal et al., 2023).

Overall, the ML-based discovery of efficient Gcc mutants proved to be helpful in optimising engineering efforts towards better and more performant carboxylases. While the hit rate of the method was relatively low, it still introduced a pair of viable mutants and reduced the amount of experimental work that would have been required otherwise to identify these variants. Coupling the outputs of the method with structural analysis also brought up a more profound and rational understanding of the results.

Discussion and Conclusions

The emission of anthropogenic CO_2 gas into the atmosphere is directly linked to the global warming crisis. Autotrophs mainly assimilate the compound naturally via the CBB cycle. However, the natural rate of CO_2 fixation is insufficient to offset the increasing amounts of emissions. Thus, in this review we described catalytically favourable synthetic pathways of inorganic carbon capturing. First, we looked at the oxygen-insensitive rGPS-MCG pathway, designed to transform pyruvate into acetyl-CoA and glyoxylate, via bicarbonate and CO_2 fixation by the Pcc and Ccr carboxylases (Luo et al., 2022). The highlight of the study consists of the self-replenishing character of the pathway, utilising a network of catalysis and supervision methods to ensure the balance of cofactors and intermediates. The cycle was, thus, able to maintain a steady state for 6 hours but was limited by the intricate instability of Ccr. The review further looked into the HOPAC cycle, constructed to generate glyoxylate from acetyl-CoA (McLean et al., 2023). While the pathway withdrew inspiration from the natural 3HP system and yielded CO_2-fixation rates comparable to the rGPS-MCG cycle, its study did not bring into discussion the need to utilise O_2-insensitive enzymes towards preventing the generation of undesired side products. At last, this review looked at another oxygen-insensitive pathway: the THETA cycle. The system was constructed to generate pyruvate from CO_2, standing out from the other pathways we described due to its modularised implementation in vivo. This was achieved via auxotrophic bacterial strains, which required the THETA cycle's products/intermediates for cell survival (Luo et al., 2023).

The current synthetic biology strategies for fixing CO_2 are varied and dependent on many factors, following diverse goals in their design (i.e. self-replenishing, in vivo integration). It is also relevant to consider designing pathways similar to natural ones, to ensure catalytic networks of orthogonal enzymes. The THETA pathway, for example, relied on the coupling of reaction steps inspired by nature, such as the 3HP cycle (also used to design the HOPAC system), and was rendered highly effective both in vitro and in vivo (Luo et al., 2023; McLean et al., 2023). Also, as the pathways we described in this review suggest, achieving high gas fixation rates with low energy (ATP) expenditure is among the critical criteria for ensuring effective systems. Fulfilling these features is among the challenges of synthetic CO_2-fixing systems. Here, the rGPS-MCG pathway proved to be the most promising cycle in ensuring effective CO_2 assimilation as per its high fixation rate, which we found to be ~10-fold higher than for the other pathways presented in this review (Table 1). However, this came at the cost of high energy expenditure. Hence, out of the systems we discussed here, the HOPAC cycle turned out to balance energy expenditure and efficiency the best.

In this review, we described the implementation of artificial carbon dioxide fixation both in vitro and in vivo. Hence, we saw that cell-free systems of CO_2 fixation are effective and feasible, and can be scaled up or modified more easily as opposed to their in vivo counterparts (Luo et al., 2022). Thus, we suggest that in vitro CO_2 synthetic assimilation is a valuable research frontier which can complement autotrophic systems at a large, perhaps industrial, scale. By feeding these systems with intermediates which drive high productivity (such as succinate in the THETA cycle) and monitoring both the cofactor balance (as seen in the rGPS-MCG cycle) and product formation with ^{13}C-labelling, these systems can sustain long-term functionality and assimilate CO_2 at an extensive scale (Luo et al., 2022; Luo et al., 2023). Although advantageous on its own, the design of in vitro CO_2-fixing systems also serves as a means for establishing in vivo pathways. The integration of synthetic pathways in cellular systems brings up a plentiful of advantages, raising the applicability of the systems (Luo et al., 2023). Here, we suggest that further research on in vivo pathways is crucial for alleviating CO_2 emissions, due to these systems' potential of assimilating the gas at large scale, in nature. Thus, by engineering microbes (such as the nonphotosynthetic *E. coli*, as seen for the THETA cycle), autotrophs and cell-free systems can be feasibly complemented towards a greener future (Luo et al., 2023; Gleizer et al., 2019; DeLisi et al., 2020). These applications could potentially also be extended to photosynthetic organisms too (i.e. plants, algae) by future research. However, it is crucial to take into account the scales of synthetic biology when it comes to its integration in societal issues. We claim that understanding the ethical challenges at the interface of these practices is critical, both for their design and the training of future synthetic biologists (Karim et al., 2024). Within synthetic CO_2-fixing systems, the assimilation of carbon molecules is performed via carboxylases. Here, we described the oxygen-insensitive phosphoenolpyruvate carboxylase and the crotonyl-CoA carboxylase/reductase enzymes as the fastest and most prominent

catalysts for fixing CO_2 and its derivatives, outcompeting RuBisCO (McLean et al., 2023). The integration of these proteins to novel pathways is not limited experimentally for the most part, since they utilise a small number of readily available cofactors and their catalysis mechanisms are generally well-understood. The Ccr enzyme, in particular, stood out due to its use in all the synthetic CO_2-fixing systems presented throughout this thesis.

Overall, the common motifs of synthetic CO_2 fixation we identified in this thesis include the integration of natural pathway segments into synthetic biocatalytic cycles. These cycles were designed in such a way that they could yield useful organic compounds (i.e. pyruvate, malate or acetyl-CoA), fixing CO_2 or its derivatives at higher/comparable rates to the native autotrophic systems. Moreover, the employment of biocatalytically efficient carboxylases was found to be relevant in the design of the cycles. Most pathways relied on utilising the crotonyl-CoA carboxylase/reductase, called the most efficient CO_2-fixing enzyme, despite not being naturally involved in this process (McLean et al., 2023; Bierbaumer et al., 2023; Erb et al., 2007). Despite all mentioned above, the goal of fully optimal artificial CO_2-fixing pathways is not reached yet. Therefore, we also studied the computational biology-based optimisation prospects of these systems.

Computational protein-studying methods have been introduced in research on the synthetic systems presented in this review, as per their role in predicting pathways and improving biocatalysts (Luo et al., 2022; McLean et al., 2023; Luo et al., 2023). Optimising synthetic CO_2-fixing pathways is greatly reliant on the design of the systems but, perhaps most importantly, on the enzymes utilised to catalyse them. Of course, it is not only the carboxylases which are responsible for the efficiency of such pathways but all the other catalysts as well. Here, we focused on carboxylases, describing how their development can depend on an in-depth understanding of underlying reaction mechanisms. The production of the C_2-adduct and the conformation-dependent reaction mechanisms in Ccr were revealed, as results of computational work in molecular dynamics (Recabarren et al., 2023; Gomez et al., 2023). These characteristics were connected with the potential of increasingly efficient protein engineering strategies for carboxylase chemistry. We also discussed the topic of computational protein development, showing how an ML-guided approach enabled the optimisation of the glycolyl-CoA carboxylase with a low hit rate (Marchal et al., 2023). Machine learning stood out as a means for artificial CO_2 fixation improvement, also due to its role in driving the METIS workflow to optimise the HOPAC and THETA cycles (McLean et al., 2023; Luo et al., 2023). Considering this, we believe that computational biology brings valuable prospects for the design and development of both enzymes and CO_2-fixing systems. Given that the caveats of the systems we described include enzyme instability and knowledge gaps with regards to alternative carboxylation routes (Pcs in the HOPAC cycle), this thesis encourages the development of reliable and effective computational methods for targeted protein development. Development of novel carbon dioxide assimilating enzymes

inspired by the features of Ccr, for example, is, further, proposed by this review as a means to diversify the pool of proteins and viable CO_2-fixing systems. Carboxylase functions in other enzyme families can also be explored, as previously researched in some reductases (Bernhardsgrütter et al., 2019). Such endeavours could sustain the efforts of shaping novel strategies for CO_2 fixation for more stable, specific and efficient biocatalytic cycles.

Overall, this thesis overviewed the current strategies in synthetic biology and suggested how computational methods can optimise both pathways and biocatalysts, particularly carboxylases. Thus, we indicated how MD simulations can be complemented by molecular modelling and AI-guided protein development to understand and better develop enzymes. We further encouraged research on both in vitro optimisation and in vivo integration of feasible, stable and effective synthetic systems, highlighting the necessity of trustworthy computational methods, and claiming the overall potential of these mechanisms to ensure a future with reduced CO_2-caused pollution.

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References

Bar-Even, A., Noor, E., Lewis, N. E., & Milo, R. (2010). Design and analysis of synthetic carbon fixation pathways. *Proceedings of the National Academy of Sciences*, *107*(19), 8889–8894. https://doi.org/10.1073/pnas.0907176107

Bernhardsgrütter, I., Schell, K., Peter, D. M., Borjian, F., Saez, D. A., Vöhringer-Martinez, E., & Erb, T. J. (2019). Awakening the Sleeping Carboxylase Function of Enzymes: Engineering the Natural CO_2-Binding Potential of Reductases. *Journal of the American Chemical Society*, *141*(25), 9778–9782. https://doi.org/10.1021/jacs.9b03431

Bierbaumer, S., Nattermann, M., Schulz, L., Zschoche, R., Erb, T. J., Winkler, C. K., Tinzl, M., & Glueck, S. M. (2023). Enzymatic Conversion of CO_2: From Natural to Artificial Utilisation. *Chemical Reviews*, *123*(9), 5702–5754. https://doi.org/10.1021/acs.chemrev.2c00581

DeLisi, C., Patrinos, A., MacCracken, M., Drell, D., Annas, G., Arkin, A., Church, G., Cook-Deegan, R., Jacoby, H., Lidstrom, M., Melillo, J., Milo, R., Paustian, K., Reilly, J., Roberts, R. J., Segrè, D., Solomon, S., Woolf, D., Wullschleger, S. D., & Yang, X. (2020). The Role of Synthetic Biology in Atmospheric Greenhouse Gas Reduction: Prospects and Challenges. *BioDesign Research*, *2020*, 1016207. https://doi.org/10.34133/2020/1016207

Diehl, C., Gerlinger, P. D., Paczia, N., & Erb, T. J. (2023). Synthetic anaplerotic modules for the direct synthesis of complex molecules from CO_2. *Nature Chemical Biology*, *19*(2), 168–175. https://doi.org/10.1038/s41589-022-01179-0

Erb, T. J. (2024). Photosynthesis 2.0: Realizing New-to-Nature CO_2-Fixation to Overcome the Limits of Natural Metabolism. *Cold Spring Harbor Perspectives in Biology*, *16*(2), a041669. https://doi.org/10.1101/cshperspect.a041669

Erb, T. J., Berg, I. A., Brecht, V., Müller, M., Fuchs, G., & Alber, B. E. (2007). Synthesis of C_5-dicarboxylic acids from C_2-units involving crotonyl-CoA carboxylase/reductase: The ethylmalonyl-CoA pathway. *Proceedings of the National Academy of Sciences*, *104*(25), 10631–10636. https://doi.org/10.1073/pnas.0702791104

Erb, T. J., & Zarzycki, J. (2018). A short history of RubisCO: The rise and fall (?) of Nature's predominant CO_2 fixing enzyme. *Current Opinion in Biotechnology*, *49*, 100–107. https://doi.org/10.1016/j.copbio.2017.07.017

Gleizer, S., Ben-Nissan, R., Bar-On, Y. M., Antonovsky, N., Noor, E., Zohar, Y., Jona, G., Krieger, E., Shamshoum, M., Bar-Even, A., & Milo, R. (2019). Conversion of Escherichia coli to Generate All Biomass Carbon from CO_2. *Cell*, *179*(6), 1255-1263.e12. https://doi.org/10.1016/j.cell.2019.11.009

Gomez, A., Erb, T. J., Grubmüller, H., & Vöhringer-Martinez, E. (2023). Conformational Dynamics of the Most Efficient Carboxylase Contributes to Efficient CO_2 Fixation. *Journal of Chemical Information and Modeling*, *63*(24), 7807–7815. https://doi.org/10.1021/acs.jcim.3c01447

Hollingsworth, S. A., & Dror, R. O. (2018). Molecular Dynamics Simulation for All. *Neuron*, *99*(6), 1129–1143. https://doi.org/10.1016/j.neuron.2018.08.011

Kai, Y., Matsumura, H., & Izui, K. (2003). Phosphoenolpyruvate carboxylase: Three-dimensional structure and molecular mechanisms. *Archives of Biochemistry and Biophysics*, *414*(2), 170–179. https://doi.org/10.1016/S0003-9861(03)00170-X

Karim, A. S., Brown, D. M., Archuleta, C. M., Grannan, S., Aristilde, L., Goyal, Y., Leonard, J. N., Mangan, N. M., Prindle, A., Rocklin, G. J., Tyo, K. J., Zoloth, L., Jewett, M. C., Calkins, S., Kamat, N. P., Tullman-Ercek, D., & Lucks, J. B. (2024). Deconstructing synthetic biology across scales: A conceptual approach for training synthetic biologists. *Nature Communications*, *15*(1), 5425. https://doi.org/10.1038/s41467-024-49626-x

Korbeld, K. T., & Fürst, M. J. L. J. (2023). Curse and Blessing of Non-Proteinogenic Parts in Computational Enzyme Engineering. *ChemBioChem*, *24*(12), e202300192. https://doi.org/10.1002/cbic.202300192

Löwe, H., & Kremling, A. (2021). In-Depth Computational Analysis of Natural and Artificial Carbon Fixation Pathways. *BioDesign Research*, *2021*, 9898316. https://doi.org/10.34133/2021/9898316

Luo, S., Diehl, C., He, H., Bae, Y., Klose, M., Claus, P., Cortina, N. S., Fernandez, C. A., Schulz-Mirbach, H., McLean, R., Ramírez Rojas, A. A., Schindler, D., Paczia, N., & Erb, T. J. (2023). Construction and modular implementation of the THETA cycle for synthetic CO2 fixation. *Nature Catalysis*, *6*(12), 1228–1240. https://doi.org/10.1038/s41929-023-01079-z

Luo, S., Lin, P. P., Nieh, L.-Y., Liao, G.-B., Tang, P.-W., Chen, C., & Liao, J. C. (2022). A cell-free self-replenishing CO_2-fixing system. *Nature Catalysis*, *5*(2), 154–162. https://doi.org/10.1038/s41929-022-00746-x

Marchal, D. G., Schulz, L., Schuster, I., Ivanovska, J., Paczia, N., Prinz, S., Zarzycki, J., & Erb, T. J. (2023). Machine Learning-Supported Enzyme Engineering toward Improved CO_2 -Fixation of Glycolyl-CoA Carboxylase. *ACS Synthetic Biology*, *12*(12), 3521–3530. https://doi.org/10.1021/acssynbio.3c00403

McCulloch, M. T., Winter, A., Sherman, C. E., & Trotter, J. A. (2024). 300 years of sclerosponge thermometry shows global warming has exceeded 1.5 °C. *Nature Climate Change*, *14*(2), 171–177. https://doi.org/10.1038/s41558-023-01919-7

McLean, R., Schwander, T., Diehl, C., Cortina, N. S., Paczia, N., Zarzycki, J., & Erb, T. J. (2023). Exploring alternative pathways for the in vitro establishment of the HOPAC cycle for synthetic CO_2 fixation. *Science Advances*, *9*(24), eadh4299. https://doi.org/10.1126/sciadv.adh4299

Meinen, B. A., & Bahl, C. D. (2021). Breakthroughs in computational design methods open up new frontiers for *de novo* protein engineering. *Protein Engineering, Design and Selection*, *34*, gzab007. https://doi.org/10.1093/protein/gzab007

Nam, K., Shao, Y., Major, D. T., & Wolf-Watz, M. (2024). Perspectives on Computational Enzyme Modeling: From Mechanisms to Design and Drug Development. *ACS Omega*, acsomega.3c09084. https://doi.org/10.1021/acsomega.3c09084

Pandi, A., Diehl, C., Yazdizadeh Kharrazi, A., Scholz, S. A., Bobkova, E., Faure, L., Nattermann, M., Adam, D., Chapin, N., Foroughijabbari, Y., Moritz, C., Paczia, N., Cortina, N. S., Faulon, J.-L., & Erb, T. J. (2022). A versatile active learning workflow for optimization of genetic and metabolic networks. *Nature Communications*, *13*(1), 3876. https://doi.org/10.1038/s41467-022-31245-z

Planas-Iglesias, J., Marques, S. M., Pinto, G. P., Musil, M., Stourac, J., Damborsky, J., & Bednar, D. (2021). Computational design of enzymes for biotechnological applications. *Biotechnology Advances*, *47*, 107696. https://doi.org/10.1016/j.biotechadv.2021.107696

Recabarren, R., Tinzl, M., Saez, D. A., Gomez, A., Erb, T. J., & Vöhringer-Martinez, E. (2023). Covalent Adduct Formation as a Strategy for Efficient CO_2 Fixation in Crotonyl-CoA Carboxylases/Reductases. *ACS Catalysis*, *13*(9), 6230–6241. https://doi.org/10.1021/acscatal.2c05250

Rosenthal, R. G., Ebert, M.-O., Kiefer, P., Peter, D. M., Vorholt, J. A., & Erb, T. J. (2014). Direct evidence for a covalent ene adduct intermediate in NAD(P)H-dependent enzymes. *Nature Chemical Biology*, *10*(1), 50–55. https://doi.org/10.1038/nchembio.1385

Santos Correa, S., Schultz, J., Lauersen, K. J., & Soares Rosado, A. (2023). Natural carbon fixation and advances in synthetic engineering for redesigning and creating new fixation pathways. *Journal of Advanced Research*, *47*, 75–92. https://doi.org/10.1016/j.jare.2022.07.011

Schwander, T., Schada Von Borzyskowski, L., Burgener, S., Cortina, N. S., & Erb, T. J. (2016). A synthetic pathway for the fixation of carbon dioxide in vitro. *Science*, *354*(6314), 900–904. https://doi.org/10.1126/science.aah5237

Sellés Vidal, L., Isalan, M., Heap, J. T., & Ledesma-Amaro, R. (2023). A primer to directed evolution: Current methodologies and future directions. *RSC Chemical Biology*, *4*(4), 271–291. https://doi.org/10.1039/D2CB00231K

Stoffel, G. M. M., Saez, D. A., DeMirci, H., Vögeli, B., Rao, Y., Zarzycki, J., Yoshikuni, Y., Wakatsuki, S., Vöhringer-Martinez, E., & Erb, T. J. (2019). Four amino acids define the CO_2 binding pocket of enoyl-CoA carboxylases/reductases. *Proceedings of the National Academy of Sciences*, *116*(28), 13964–13969. https://doi.org/10.1073/pnas.1901471116

Vögeli, B., Rosenthal, R. G., Stoffel, G. M. M., Wagner, T., Kiefer, P., Cortina, N. S., Shima, S., & Erb, T. J. (2018). InhA, the enoyl-thioester reductase from Mycobacterium tuberculosis forms a covalent adduct during catalysis. *Journal of Biological Chemistry*, *293*(44), 17200–17207. https://doi.org/10.1074/jbc.RA118.005405

Zarzycki, J., & Fuchs, G. (2011). Coassimilation of Organic Substrates via the Autotrophic 3-Hydroxypropionate Bi-Cycle in Chloroflexus aurantiacus. *Applied and Environmental Microbiology*, *77*(17), 6181–6188. https://doi.org/10.1128/AEM.00705-11