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A synthetic pathway for the fixation of carbon dioxide in vitro

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Abstract

Carbon dioxide (CO₂) is an important carbon feedstock for a future green economy. This requires the development of efficient strategies for its conversion into multi-carbon compounds. Here, we describe a synthetic cycle for the continuous fixation of CO₂ in vitro. The crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle is a reaction network of 17 enzymes that converts CO₂ into organic molecules at a rate of 5 nmol CO₂ min⁻¹ mg⁻¹ protein. The CETCH cycle was drafted by metabolic retrosynthesis, established with enzymes originating from nine different organisms of all three domains of life and optimized in several rounds by enzyme engineering and metabolic proofreading. The CETCH cycle expands the six naturally evolved CO₂-fixation pathways by a seventh, synthetic alternative, opening the way for in vitro and in vivo-applications.

Every year more than 350 gigatons CO₂ are transformed by Nature in a sustainable process called autotrophic CO₂-fixation. More than 90% of the carbon is fixed by the Calvin-Benson-Bassham (CBB) cycle in plants, algae and microorganisms. The rest is converted through alternative autotrophic CO₂-fixation pathways (1, 2). Despite this naturally existing diversity, the application of CO₂-fixing enzymes and pathways for converting CO₂ into multi-carbon, value-added products has been limited so far in chemistry (3, 4) and biotechnology (5). First, natural CO₂-fixation delivers mainly biomass and not a dedicated product. Moreover, under optimal conditions biological CO₂-fixation is often directly affected by the inefficiency of the CO₂-fixing enzymes and pathways. The CBB cycle's carboxylase ribulose-1,5-bisphosphate carboxylase/oxidase (RubisCO) for instance is a slow catalyst that shows a strong side reaction with oxygen, which leads to the loss of fixed carbon and thus photosynthetic energy by up to 30% in a process called photorespiration (6).

There have been several attempts to improve biological CO₂-fixation (7), including evolving RubisCO towards higher reaction rate and specificity (8, 9), engineering more efficient photorespiration (10, 11) or transplanting natural CO₂-fixation pathways into non-

Author Contributions

T.J.E. conceived the project. T.J.E., L.S.v.B. and T.S. designed the experiments, analysed the data and wrote the manuscript. T.J.E., T.S. and L.S.v.B. performed experiments, and S.B. assisted in experiments. N.S.C. performed mass spectrometry and analysed the data.

autotrophic organisms, such as *E. coli* (12–14). In contrast to these efforts, which showed only limited success, the emerging field of synthetic biology provides an alternative approach to create designer CO₂-fixation pathways. By freely combining different enzymatic reactions from various biological sources, completely artificial CO₂-fixation routes can be constructed that are kinetically or thermodynamically favored compared to the naturally evolved CO₂-fixation pathways. Several synthetic routes for CO₂-fixation have been theoretically considered (15). Yet, none of these artificial pathways could be realized so far, emphasizing an existing gap between theoretical design and experimental realization in synthetic biology. A common problem is the attempt to directly assemble synthetic pathways in living organisms. These efforts are challenged by the lack of understanding the complex interplay between the different enzymes used in these synthetic networks, as well as the interference of the synthetic networks in the complex background of the host organism, causing undesired effects like side-reactions and toxicity. Therefore the realization of synthetic pathways requires novel strategies that first allow their testing and optimization in more defined conditions (16–19). To overcome these limitations, we decided to take a radically different, reductionist approach by assembling a synthetic CO₂-fixation cycle from its principal components in a bottom-up fashion (Fig. S1).

A known bottleneck in natural CO₂-fixation is the efficiency of a carboxylating enzyme in a given pathway (20–22). To identify a suitable CO₂-fixation reaction for our synthetic cycle we first compared the different biochemical and kinetic properties of all known major carboxylase classes (23) (Table S1). Based on this analysis, we decided to rely on coenzyme A (CoA)-dependent carboxylases and in particular enoyl-CoA carboxylases/reductases (ECRs) due to their favorable catalytic properties. ECRs are a recently discovered class of carboxylases that operate in secondary metabolism, as well as in central carbon metabolism of Alphaproteobacteria and Streptomyces, but notably not in any autotrophic CO₂-fixation pathway, known so far (24). Compared to RubisCO and other carboxylases, ECRs span a broad substrate spectrum (25), are oxygen-insensitive, do not accept molecular oxygen as substrate, only require the ubiquitous cofactor NADPH, and catalyze the fixation of CO₂ with high catalytic efficiency (i.e., on average between two- to four-fold better than RubisCO, Table S1) (26).

We conceived several theoretical CO₂-fixation routes that (i) start with a given ECR reaction, (ii) regenerate the carboxylation substrate to allow for a continuous cycling, and (iii) feature a dedicated output reaction to channel the fixed carbon into a product (Fig. S1). In contrast to earlier approaches (15, 27), we did not restrict our design to known enzymes, but considered all reactions that seemed biochemically feasible (i.e., fall into one of the standard reaction classes defined by the Enzyme Commission). We obtained several prospective cycles of which seven were considered further (Fig. S2A-G).

In the following, we evaluated the thermodynamic feasibility of these theoretical cycles by calculating their Gibbs free energy profile ($\Delta_r G'$) under biologically relevant conditions and estimating the presumable ATP- and NADPH-consumption of each cycle per CO₂-molecule converted (Fig. S2, Supplementary Text). Our calculations showed that the synthetic cycles were generally more energy-efficient compared to a stand-alone CBB cycle, as well as the core sequence of other naturally existing, oxygen-insensitive CO₂-fixation pathways, such as

the 3-hydroxypropionate (3HP) bicycle and the 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle (Table S2). As an example, four of our synthetic cycles required on average 1/3 less ATP per CO₂ converted into phosphoglycerate compared to the core sequence of the CBB cycle. This would translate to at least seven photons less per phosphoglycerate formed from CO₂ in a theoretical photosynthetically-coupled process (Table 1, Fig. S3 & S4). In summary, these calculations suggested that our synthetic cycles could provide very promising solutions to an efficient C-C bond formation under aerobic conditions that compete with naturally existing metabolic pathways.

Next, we searched bioinformatic databases for enzymes that could sustain our drafts (Fig. S2). For the CETCH cycle (Fig. S2A) we were able to identify one or more potential enzyme candidates for each reaction of its core sequence. We purified, tested and characterized these candidates and selected a set of 12 enzymes, each with a specific activity >1 U mg⁻¹ protein (at 250 μM substrate concentration, (Table S3), to experimentally realize the CETCH cycle as a proof of principle. To demonstrate feasibility of the CETCH cycle, we first reconstituted its central CO₂-fixation reaction sequence stepwise. We provided cofactors and the chemically synthesized intermediate propionyl-CoA in a buffer containing NaHCO₃. To this mixture, propionyl-CoA carboxylase (Pcc) was added first, followed by the subsequent addition of the other eleven enzymes. HPLC-MS analysis demonstrated the stepwise transformation of propionyl-CoA into key intermediates of the pathway (Fig. S5), indicating that this first version of the CETCH cycle (CETCH 1.0) was in principle functional.

To test for continuous operation of CETCH 1.0, we provided all enzymes, cofactors and the artificial electron acceptor ferrocenium from the beginning and started the cycle by addition of propionyl-CoA (Fig. S6A). To follow the dynamics of the cycle, we used NaH¹³CO₃ as CO₂ source for this and all subsequent experiments. Due to the constant incorporation of CO₂ during the course of the cycle, enrichment of the ¹³C-isotope in the intermediates can be used to follow flux through the CETCH cycle in the continuous mode and under apparent steady-state conditions (Fig. S7). However, the conversion of CoA-esters stopped before a complete turn of the cycle at the level of methylsuccinyl-CoA, indicating that the methylsuccinyl-CoA dehydrogenase (Mcd) (28) enzyme reaction used in the cycle was rate limiting (Fig. S6B-C, Supplementary Text).

Mcd belongs to the family of FAD-dependent acyl-CoA dehydrogenases, which are coupled via electron transfer flavoproteins to the membrane bound ubiquinone pool and eventually to the respiratory chain. In the stepwise reconstitution of CETCH 1.0 we had added the artificial electron acceptor ferrocenium together with Mcd to substitute the electron transfer cascade. Under continuous conditions, the use of the artificial electron acceptor from the beginning was limited to 0.1 mM, because higher concentrations caused other enzymes in the assay to precipitate. At 0.1 mM, however, the spontaneous reduction of ferrocenium to ferrocene by NADPH (29), as well as the slow re-oxidation rate of ferrocene most probably prevented effective cycling of CETCH 1.0.

To overcome the limitations caused by Mcd, we engineered the enzyme to directly use molecular oxygen as electron acceptor. Based on structural considerations (Fig. 1,

Supplementary Text), we introduced three point mutations (T317G, E377N, W315F) to convert the dehydrogenase into a functional methylsuccinyl-CoA oxidase (Mco) that catalyzes the oxygen-dependent oxidation of methylsuccinyl-CoA with a v_{max} of 97 ± 6 mU mg^{-1} and an apparent K_M of 27 ± 5 μM . In version 2.0 of the CETCH cycle, we replaced Mcd with the engineered Mco. Fractional labeling of intermediates showed that CETCH 2.0 was at least turning twice within 45 minutes (Fig. S8).

After successful demonstration of the CETCH core cycle under continuous conditions, we next optimized its reaction sequence. We established a read-out module to directly quantify CO_2 -fixation (Fig. S9A). Starting from propionyl-CoA, the CETCH cycle converts two molecules of CO_2 per turn into one molecule of glyoxylate. To measure glyoxylate formation we used a malate synthase (Mas) from *Escherichia coli*, which condenses glyoxylate with externally added acetyl-CoA to yield malate. From here on we used the malate production rate as a readout – in combination with CoA ester analysis – to improve CO_2 -fixation efficiency of the CETCH cycle stepwise (CETCH 2.0-5.4, Supplementary Text 2).

During the optimization phase, we added enzymes for ATP- and NADPH-regeneration, provided chemical and enzymatic protection against oxidative damage from H_2O_2 -producing enzymes and took care of unwanted side reactions that prevented effective cycling. The CETCH cycle includes enzymes from very different biological sources. The combination of such diverse enzymes into a synthetic pathway poses the challenge that these enzymes face metabolites to which they were never exposed in their native metabolic context, causing undesired side-reactions. Inspired by biology, we pursued different strategies to overcome this problem (Supplementary Text 2, Fig. S9-S12). We replaced the original pathway design with alternative reaction sequences, used enzyme engineering to minimize side reactions of promiscuous enzymes, and introduced proofreading enzymes to correct for the formation of dead-end metabolites. While the first two strategies are applied in biotechnology and synthetic biology to improve productivity (30, 31), the concept of metabolic proofreading was only considered in few cases for synthetic pathway design (16) although proofreading apparently exists in naturally evolved pathways (32). We believe that this design principle should be included more systematically in metabolic engineering in the future.

Over the course of the optimization, CO_2 -fixation efficiency in the CETCH cycle improved by almost a factor of 20 until version 5.4 (Fig. 2). CETCH 5.4 represents an in vitro enzyme network that is able to fix CO_2 at a rate of $5 \text{ nmol min}^{-1} \text{ mg}^{-1}$ of core cycle proteins (Fig. 2C). This is comparable to the few reported attempts to measure the CBB cycle in cell extracts ($1\text{--}3 \text{ nmol min}^{-1} \text{ mg}^{-1}$ CBB cycle protein, assuming that CBB enzymes account for 30% of the proteome) (33). However, we note that cell extracts represent a more complex environment compared to our reductionist in vitro system.

In total, the 13 core reactions of CETCH 5.4 together with the auxiliary proofreading and cofactor regeneration processes are catalyzed by 17 enzymes from nine different organisms and all three domains of life, including bacteria, archaea, plants and humans. In addition, the cycle features three reactions that were created by rational active-site engineering of existing

enzyme scaffolds to catalyze the desired activities, such as Mco and Pco. Notably, CETCH 5.4 relies solely on the reductive carboxylation of enoyl-CoA esters. Although ECRs belong to the most efficient CO₂-fixing enzymes known to date, they were not employed by nature for autotrophic CO₂-fixation during evolution, as far as we know. The reallocation of reductive carboxylation as key reaction for a synthetic autotrophic CO₂-fixation cycle thus goes beyond simply improving or reshuffling naturally existing autotrophic CO₂-fixation reactions and pathways. Thus, the CETCH cycle expands the diversity of the six naturally evolved CO₂-fixation strategies by a seventh, synthetic alternative that did not require the serendipity of evolution to bring together all components in space and time (34).

The successful in vitro reconstitution of a synthetic enzymatic network for the conversion of CO₂ into organic products that is superior to chemical processes and competes with naturally existing CO₂-fixing solutions in vitro opens the door for several future applications. These could include the in vivo-transplantation of synthetic CO₂-fixation cycles into lithotrophic or photosynthetic organisms, paving the way to improved CO₂-fixation (35); the use of synthetic CO₂-fixation cycles in the development of artificial photosynthetic processes (e.g., in combination with photovoltaics or artificial leaves)(36) ; or as starting point for the design of a self-sustained, completely synthetic carbon metabolism in artificial or minimal cells (37).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Structure guided engineering of Mcd into a Mco.

(A) Active site comparison of the human short-chain acyl-CoA dehydrogenase (PDB: 2VIG, green backbone) with a model of Mcd from *Rhodobacter sphaeroides* (blue backbone, modeled by the SWISS-MODEL server with 2VIG as template) and the short-chain acyl-CoA oxidase 4 from *Arabidopsis thaliana* (PDB: 2IX5, orange backbone). The three residues that were targeted to introduce oxidase activity into Mcd are highlighted. (B) HPLC-MS based analysis of wild-type and different single active-site mutants for oxidation of methylsuccinyl-CoA into mesaconyl-CoA with molecular oxygen as electron acceptor. The screen identified three substitutions (W315F, T317G and E377N) that were combined into double and triple mutants. (C) Michaelis-Menten graphs of single, double and triple mutants characterized in more detail. The triple mutant showed best kinetic parameters.

CO₂-fixation efficiency is defined as the CO₂-equivalents fixed per acceptor molecule in the cycle (i.e., starting amount of propionyl-CoA). Right x-axis: Absolute malate concentration formed over the course of 90 minutes in CETCH 5.4. The final assay (0.52 ml) contained 2.3 mg ml⁻¹ protein of cycle core enzymes plus 0.8 mg ml⁻¹ auxillary enzymes and produced 540 μM malate over 90 min, which corresponds to 1080 μM fixed CO₂.

Table 1
Comparison of selected theoretical and synthetic CO₂-fixation cycles with the core sequence of naturally existing, aero-tolerant CO₂-fixation pathways.

Shown are the number of reaction steps, the number of NAD(P)H, ATP and FADH₂ required (red) or generated (blue) during conversion of CO₂ into one molecule of phosphoglycerate. These values were normalized to ATP equivalents required per CO₂ molecule fixed into phosphoglycerate, or alternatively to the number of photons per CO₂ fixed into phosphoglycerate in a theoretical photosynthetically-coupled process (see Supplementary Text 1 and Fig. S2 & S3 for a more detailed analysis).

Pathway	Status	# rxns ^[a]	$\Sigma_{\text{NAD(P)H}}$	Σ_{ATP}	Σ_{FADH_2}	ATP equiv. per CO ₂ ^[b]	Photons per CO ₂ (additional ATP left) ^[c]
CETCH cycle ^[d]	Theoretical	11				-6.2	16 (+ 2.1 ATP)
HOPAC cycle ^[d]	Theoretical	12				-6.5	16 (+ 1.1 ATP)
FUMES cycle ^[d]	Theoretical	11				-6.5	16 (+ 2.1 ATP)
HITME cycle ^[d]	Theoretical	12				-6.5	16 (+ 2.1 ATP)
CBB cycle	Natural	13			0	-6.8	21 (+0.1 ATP)
CBB cycle (+ O ₂) ^[e]	Natural	13			0	-8.2	24 (n.c.)
3HP bicycle ^[f]	Natural	15				-6.8	24 (+0.7 ATP)
3HP/4HB cycle ^[g]	Natural	13				-8.0	28 (+1.0 ATP)
CETCH 2.0 – 4.0 ^{[d],[h]}	Synthetic	11			0 / 1 ^[i]	-7.5 / -6.5 ^[i]	16 (+2.1 ATP)
CETCH 5.0 – 5.4 ^{[d],[h]}	Synthetic	13			0 / 2 ^[i]	-8.5 / -6.5 ^[i]	20 (+4.4 ATP)

^[a] Stereochemical conversions, such as racemization or epimerization reactions are not taken into account.

^[b] For ATP per CO₂ conversion a P/O ratio of 2.5 for NAD(P)H and 1.5 for FADH₂ was assumed (38).

^[c] For photons per CO₂ conversions, the number of biologically generated photons required for the generation of the NADPH and ATP in each cycle was calculated, assuming a stoichiometry of 1 NADPH and 1.28 ATP per 4 photons (39). The photon energy left is given in ATP equivalents. These calculations do not consider the additional generation of ATP through any FADH₂ generated through a given pathway.

^[d] Transformation of the initial CO₂-fixation product glyoxylate into phosphoglycerate was calculated via glyoxylate carboligase (40).

^[e] Assuming a loss of 20% photosynthetic energy due to photorespiration.

^[f] Transformation of the initial CO₂-fixation product pyruvate was calculated via pyruvate synthase.

^[g] Transformation of the initial CO₂-fixation product acetyl-CoA via pyruvate: water dikinase

^[h] Transformation of the initial CO₂-fixation product acetyl-CoA via acetyl/propionyl-CoA carboxylase and PEP carboxykinase.

^[i] Calculations for a process, where the electrons from the two oxidase reactions could be conserved via a respiratory chain.